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Journal of DENTAL RESEARCH

Journal of DENTAL RESEARCH

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This *Journal* is dedicated to the dissemination of knowledge pertaining to the oral cavity, its contents and contiguous parts, and its relation to and effect on the total human organism. The *Journal* publishes original investigations in these and related fields. Articles are accepted only if they are contributed solely to this *Journal*.

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Editorial Views and News

More and more journals in the area of the sciences are utilizing *Interlingua* in "summarios" of published papers. There is little doubt that Interlingua is a practical and useful adjunct to *Communication in le scienta*. Since it can be read almost completely on first sight by every educated person, it can be truly called *Interlingua—servitor del scientia*.

The Editor of the *Journal of Dental Research* earnestly seeks the opinions of readers regarding the use of *Interlingua* in summaries or abstracts of manuscripts to be published in *JDR*. There appears to be a considerable trend toward the use of this significant tool in international communication. To demonstrate how simple it is for most people to comprehend these words, readers ought to try the following:

Interlingua—Le libore circulation de ideas e informationes es non minus importante pro le scientia que le circulation del sanguine lo es pro le le corpore. Sed iste circulation nunc es impedite per le publication del resultatos de recercas scientific in un multiplicitate de linguas. Le recente reporto de UNESCO super le "Traduction Scientific e Technic e Altere Aspectos del Problema de Lingua" estima que al minus 50 pro cento del litteratura scientific del mundo es publicate in linguas que plus que 50 pro cento del scientistas del mundo non pote leger.

IADR Biographical Directory.—Inquiries are continually being made about revising the Biographical Directory of IADR members which appeared in the *Journal* eight years ago. Although this may be a somewhat formidable task, there appears to be real need for such a directory. Very little is known of our younger members and those from overseas. Thus, as suggested in "Editorial Views and News" of January–February, 1959, in *JDR*, only those members might be included that are not listed in the tenth edition of *American Men of Science* (now being produced); the others could be listed by name and address only. The Editor would like to hear from IADR members on this matter of a revised directory as a supplement to the *Journal*.

Medico-legal Conference.—Another National Conference on the Legal Environment of Medical Sciences is to be held, though the exact time and site have not yet been determined.

It is not at all amazing to realize that research, even though confined to the apparently restricted, though not isolated, area of the oral cavity, does have legal involvements of several kinds.

At the first Conference on Legal Environment sponsored by the National Society for Medical Research held at the University of Chicago Clinics and attended by the *JDR* Editor, a great variety of problems was thoroughly discussed. In the full report now available, such topics were considered as organ transplants, autopsy procedures, and bequests for all or parts of bodies for science. In a second session, the care and use of laboratory animals was considered, as well as legal and ethical factors regarding animal experimentation. Problems of consent in clinical investigation were considered in a third session. Herein research on human beings was discussed, with special consideration to drug testing. In the oral area, there have been innumerable occasions for the clinical testing of new drugs, although to some extent the pharmacological action may have been on a local basis.

Experimental design.—In planning a research project concerning the oral area with its multiplicity of experiments, some simple but most extremely complex, there is no doubt that the prospective researcher must think clearly and very comprehensively about principles which underlie the acquisition of new basic information. The research worker must realize that control of the natural things comes only with absolutely accurate knowledge about the macro- and/or microenvironment concerned. Methods used for searching out natural phenomena are certainly diversified. In those instances wherein the experimental method is used, one must, in a restricted sense, control the environment of the region for a definite time period. The mode of most logically consummating this task comes under the significant topic of "experimental design."

Proper experimental design is paramount in research these days, as is evidenced by its lack in many manuscripts submitted to *JDR*. If the experimental design of a study concerning the oral area is inadequate, then at the end of perhaps a long and costly project, one has not had an experiment at all, but merely an experience!

—F. J. O.

INTERNATIONAL ASSOCIATION FOR DENTAL RESEARCH

Thirty-ninth Annual Meeting, March 23, 24, 25, and 26, 1961
Hotel Statler, Boston, Massachusetts

Abstracts of papers to be considered for presentation at the thirty-ninth annual meeting of the I.A.D.R. must be submitted on special forms provided by the Association and must be in the hands of the chairman of the program committee not later than December 1, 1960.

Preprinted abstracts for distribution prior to the meeting will be prepared photographically from abstracts on the special forms. Abstracts not on the forms cannot be accepted. Abstracts must fulfil the requirements stated on the forms and must be fully ready, without editing, for inclusion in the preprinted abstracts. If time allows, abstracts not suitable will be returned to the authors.

The program committee will select papers for presentation at the meeting on the basis of the quality of the work as judged from the abstract and according to whether the presentation will fit into the plan of the meeting as determined by the groupings of papers submitted.

Abstracts of papers presented at the meeting will be formally published in the *Journal of Dental Research* after the meeting. Revisions may be made prior to formal publication by arrangement with the editor.

Authors who qualify as novices may compete for the Edward H. Hatton Award at the meeting. Please write to the chairman of the Hatton Award Committee for information: Dr. Roy O. Greep, 188 Longwood Avenue, Boston 15, Massachusetts.

To obtain forms for abstracts, write to: Howard F. Campbell, Assistant Secretary-Treasurer, 2000 P Street, N.W., Washington 6, D.C.

Send abstracts on the forms provided to: Dr. James A. English (Program Chairman), School of Dentistry, University of Buffalo, Buffalo 14, New York.

Deadline for receipt of abstracts—December 1, 1960.

The Effect of a Commercial Stannous Fluoride Dentifrice under Controlled Brushing Habits on Dental Caries Incidence in Children: Preliminary Report

GEORGE E. PEFFLEY and JOSEPH C. MUHLER
Indiana University, Bloomington, Indiana

In much of the clinical testing[1–4] which has been conducted to evaluate a stannous fluoride–calcium pyrophosphate dentifrice as an anticaries agent, it was found that the participating children brushed their teeth on the average of less than once a day. However, in a clinical test among young adults[5] it was found that many of the subjects brushed quite frequently. Those who brushed with the stannous fluoride dentifrice three or more times per day appeared to receive greater benefit from the dentifrice than did those who brushed less frequently. While the random-brushing tests showed significant reductions in caries, it was reasonable to expect greater protection from more frequent applications of the stannous fluoride dentifrice.

The objective of this study was to measure the effectiveness of the test product when both test and control products were used three times per day under supervision.

EXPERIMENTAL METHODS

This test was conducted in a military school where all the boys lived under almost identical conditions. The age of the subjects ranged from ten to nineteen years. All boys received a thorough clinical and radiographic examination at the beginning of the study, and, from the combined results, the subjects were divided into two groups upon the basis of past dental caries history. Group I received a stannous fluoride–stannous pyrophosphate dentifrice and Group II the identical dentifrice minus the stannous fluoride and stannous pyrophosphate. The compositions of these dentifrices are shown in Table 1.

The test was designed to extend over the period of 2 school years, with a supply of the product being provided over the summer vacation. Because of the nature of the population under study, it was most convenient to perform the examinations at the end of each school term. Thus examinations were to be performed approximately 5, 10, and 22 months after the start of the test. This report covers the results of the 5- and 10-month examinations. These periods were shorter than desired but represented the only realistic compromise possible with the groups used. The results of the whole 2-year study will be reported at the end of the test period.

This study was supported in part by a grant from the Procter and Gamble Company, Cincinnati, Ohio.

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All the children were requested to brush their teeth at least 2 minutes per brushing three times a day. Subsequent unannounced check examination of their oral hygiene showed clearly that good brushing habits were being practiced. Also, the improvement in their oral hygiene at the 5- and 10-month clinical re-examinations similarly indicated an improvement in their brushing habits. Evidence indicated that almost all the children brushed three times a day—once following breakfast and dinner and usually before retiring.

After 5 and 10 months following the initial examination the children were re-examined and radiographed by the same dentist. The conduct of the study and the examinations themselves were conducted in accordance with the conditions previously practiced and described for similar clinical studies.[6]

RESULTS AND DISCUSSION

The average age at the beginning of the test was identical for both the experimental (Group I) and the control (Group II) groups, each being fourteen years and ten months. The average initial DMFS was 25.56 for the children in the experimental

TABLE 1

COMPOSITION OF DENTRIFRICES USED IN THIS STUDY

Dentifrice Constituent	Control (Per Cent)	Experimental (Per Cent)
Calcium pyrophosphate	40.00	39.00
Detergent (non-soap)	1.51	1.51
Humectant	30.00	30.00
Binder	1.55	1.55
Stannous pyrophosphate	.....	1.00
Minor ingredients (flavoring agents)	1.44	1.44
Stannous fluoride	.....	0.40
Water	25.5	25.10
pH	6.5	4.8

group and 24.75 for the children in the control group. Table 2 shows the age and the number of children at the initial, 5-month, and 10-month examination periods.

Table 3 shows the results at the end of the 5- and 10-month periods in terms of average increments of DMFT and DMFS, along with the number of subjects, the percentage reduction in the dental caries rate, and the probability. The data obtained at the end of the 5-month period demonstrate a reduction in dental caries in terms of either the DMFT or the DMFS index. Similarly, at the end of the 10-month period the data show a highly significant anticariogenic effect. These data demonstrate that the effect of the stannous-fluoride dentifrice is effective in reducing the dental caries experience and that it maintains this degree of effectiveness over the entire 10-month period.

An interesting point about the relative values of the DMFS and DMFT indexes is illustrated by these data. Table 3 indicates that the stannous fluoride dentifrice completely prevented the development of dental caries when the results are expressed by the DMFT index. That this indication is erroneous is demonstrated by the DMFS data, which show that the subjects using the stannous fluoride developed 84 new cari-

ous surfaces. While this represents a significant reduction of 58 per cent in tooth decay, it would be improper to represent the result of this test by using only the DMFT index. One would conclude from this study that the DMFS index offers a more complete and accurate representation of test results than does the DMFT index. Furthermore, if one wishes to express a test result by means of only one index, it appears that the DMFS index would have to be the choice.This is in agreement with the analyses[7] of caries indexes made in a report of a conference to develop uniform standards and procedures in clinical studies of dental caries, published by the American Dental Association in 1955.

TABLE 2

AGE AND NUMBER OF CHILDREN IN EXPERIMENTAL AND CONTROL GROUP AT INITIAL, 5-MONTH, AND 10-MONTH EXAMINATION PERIODS

AGE AT START OF TEST	NUMBER OF SUBJECTS					
	Available at Start		After 5 Months		After 10 Months	
	Control	Experimental	Control	Experimental	Control	Experimental
10	9	5	9	5	9	4
11	7	10	7	10	7	9
12	10	16	10	16	10	16
13	21	21	21	21	21	21
14	31	29	31	29	30	29
15	27	33	27	33	28	33
16	28	24	28	24	27	21
17	21	18	21	17	19	17
18	3	4	3	4	4	4
19	.....	2	.....	.....	.....	.....
Total	157	162	157	161	155	156

TABLE 3

AVERAGE INCREASE IN DENTAL CARIES EXPERIENCE IN 10- TO 19-YEAR-OLD CHILDREN USING STANNOUS FLUORIDE (GROUP I) OR CONTROL (GROUP II) DENTIFRICE THREE TIMES A DAY

	Group	No. of Subjects	DMFT	DMFS	Reversals
5-month period	I	161	−0.01	0.52	1.30
	II	157	0.36	1.25	1.06
10-month period	I	156	0.05	0.878	1.79
	II	155	0.78	2.025	1.18
Per cent reduction in caries rate	5-month	.....	103	58.4	.....
	10-month	.....	93	57.0	.....
Probability	5-month	.....	0.02	0.02	.....
	10-month	.....	0.0003	0.0034	.....

SUMMARY

The controlled use of a commercially available stannous fluoride dentifrice reduced the incidence of dental caries in ten- to nineteen-year-old children residing in a military school who brushed their teeth three times a day throughout a 10-month period. This result is thought to be related to the greater frequency of usage of the product in this test than in those conducted previously with a similar product.

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Factors Which Influence the Microbiological Response to Saliva

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Microbiological assay techniques[1, 2] have proved valuable in the examination of saliva for vitamins and amino acids.[3, 4, 5] Inasmuch as the interest in saliva is in its content of free stimulatory compound the application of these analytical methods did not appear to present any particular problems in regard to special preparatory treatment of the samples.[5] It has therefore been customary to use the more or less standard and proved assay procedures.[3, 5] However, those who have used microbiological assay methods readily appreciate that small variations in procedure may sometimes exert profound effects upon the response of the test micro-organism.[6]

With these considerations in mind some of the initial technical steps of the microbiological assay were examined in an effort to improve the application of this method of analysis to saliva. By the very nature of the test, any modification that does not violate the principles of the microbiological assay but which will result in a greater growth response of the test micro-organism lends improvement to the procedure. These considerations relate to the recognition and elimination of artifacts which are likely to develop as the result of manipulating any biological material and which must be appreciated when evaluating a study of such material.

The results given in this report are not presented in terms of a microbiological assay but rather in terms of growth response. It should be noted, however, that growth curves may be used as typical assay results by comparing growth responses at any given interval. To evaluate the assay procedure the effect of autoclaving and dilution on the growth response to saliva and its crude fractions has been examined.

EXPERIMENTAL METHODS

The test micro-organism used was *Lactobacillus casei*, strain L652, originally obtained from the Zoller Dental Clinic, University of Chicago. Stock cultures were maintained on slants of tomato juice–yeast extract medium.

The double-strength assay medium employed was of the following composition: glucose, 20 gm.; casamino acids,† 10 gm.; *L*-cystine, 20 mg.; *L*-tryptophane, 10 mg.; *L*-asparagine, 50 mg.; adenine, 20 mg.; guanine, 20 mg.; xanthine, 20 mg.; uracil, 20

This investigation was supported in part by Research Grants D-181(C), and D-181(C2), and D-181(C3) from the N.I.D.R., U.S. Public Health Service, and in part by contributions from the dental profession of the state of Oklahoma.

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* Present address: Research Corporation, San Francisco, California.

† An acid hydrolysate of casine obtained from the Difco Laboratories, Inc.

mg.; "Tween 80," 0.5 ml.; pyridoxal, 100 μg.; riboflavin, 500 μg.; nicotinic acid, 500 μg.; thiamin, 500 μg.; calcium pantothenate, 500 μg.; biotin, 10 μg.; sodium acetate, 12 gm.; K_2KPO_4, 2 gm.; KH_2PO_4, 2 gm.; $MgSO_4 \cdot 7H_2O$, 250 mg.; NaCl, 20 mg.; $FeSO_4 \cdot 7H_2O$, 20 mg., and $MnSO_4 \cdot H_2O$, 14 mg. The final pH was 6.8. In this medium, folic acid partially replaced the activity of saliva.

Whether the glucose should be autoclaved separately or in the basal medium apparently depends on the organism and the medium employed. A number of reports have indicated that certain lactic acid bacteria will not commence to grow or, at best, grow only very slowly if the glucose is autoclaved in the medium.[7, 8] Other investigators have demonstrated opposite results.[9, 10] Since the test organism in the medium used in this study showed a more rapid rate of growth and a greater total growth when the glucose

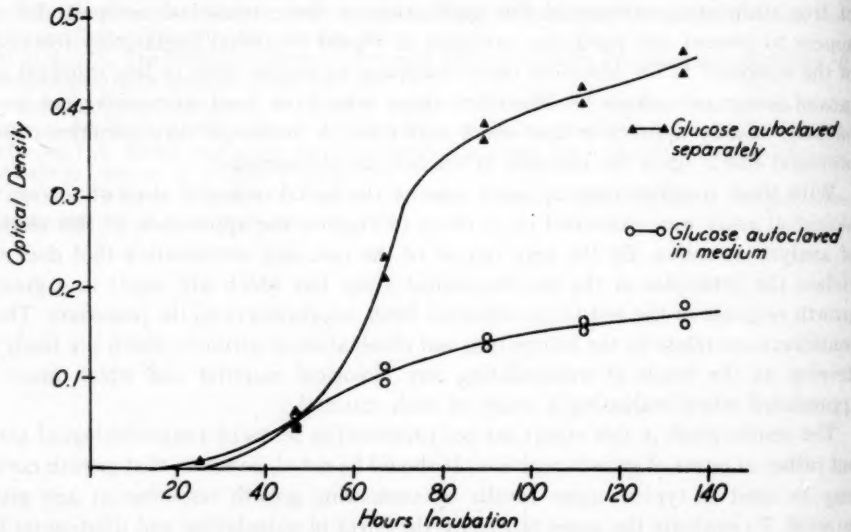

FIG. 1.—Growth response of an oral lactobacillus in a basal medium supplemented with 0.002 μg. folic acid/ml, demonstrating the effect of autoclaving glucose separately as a 10 per cent solution in the basal medium.

was autoclaved separately (Fig. 1), the glucose was sterilized as a 10 per cent solution and added aseptically to the sterile basal medium to give a final concentration of 1 per cent.

It was initially observed that smooth reproducible assay curves demonstrating a response proportional to the concentration of saliva, were not obtained with the above medium when the inoculum consisted of 0.1 ml. of a 1:1000 dilution of a twice-washed 24-hour culture of the test micro-organism grown in a nutritionally complex, tomato juice–yeast extract, broth medium. This difficulty was overcome when the inoculum was prepared by incubating the test micro-organism in the same basal medium as used for the assay, but without glucose, and supplemented with 0.5 μg. folic acid/ml.

Approximately 15 ml. of paraffin-stimulated saliva specimens were collected in large test tubes chilled with ice and maintained at about 5° C. until the tests were performed. The time lapse between collection and use of the saliva was usually not more than 2 hours.

Following the appropriate treatment of saliva (autoclaving, centrifugation, and/or dilution with the double-strength medium or water) graded amounts* were added to 18 × 150 mm., optically matched test tubes that contained 5 ml. of the double-strength medium. Redistilled water was then added to give a volume of 9 ml. The tubes were covered with aluminum caps and autoclaved at a pressure of 15 pounds for 10 minutes. After cooling, 1 ml. of a 10 per cent autoclaved, glucose solution was added aseptically to provide a 1 per cent level of glucose and a total volume of 10 ml. per tube. In the case of those experiments in which the saliva was sterilized separately (not in the medium), the double-strength medium was diluted with the appropriate amount of water to accommodate the amounts of saliva to be added, was autoclaved and cooled, and the sterile saliva and glucose were added aseptically and the tubes inoculated. The growth response was determined turbidimetrically at 540 mμ on a colorimeter†

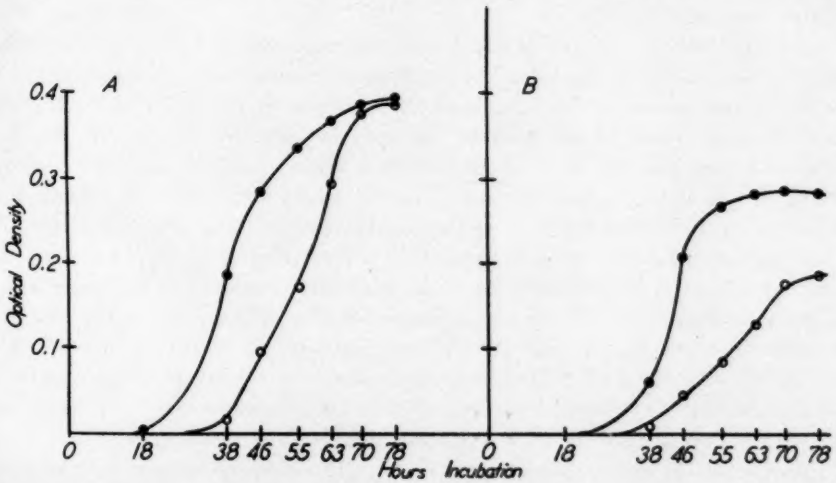

FIG. 2.—Growth response of an oral lactobacillus to (*A*) 0.5-ml. and (*B*) 0.25-ml. aliquots of a salivary sample autoclaved separately before addition to the basal medium, ●—●, and autoclaved in the basal medium minus glucose, ○—○. (Final volume 10 ml.)

at intervals during a period of incubation. The period of incubation was, in general, determined by the time required for the test micro-organism to attain maximum growth in a given saliva-medium mixture. Maximum growth could be recognized when continued incubation did not result in an increased optical density of the culture.

RESULTS

Effect of autoclaving saliva in the basal medium.—To determine whether autoclaving saliva in the basal medium resulted in any changes that would affect the results of a microbiological assay, aliquots of the same salivary samples were autoclaved both separately and in combination with the basal medium. Saliva autoclaved separately

* Usually, the response to from 1 to 10 per cent saliva was tested, and the concentration that best demonstrated the effect of the technical procedures examined was used to illustrate the contrasting effects in the accompanying figures.

† Bausch and Lomb Spectronic 20.

and added aseptically to the basal medium was found to effect a shorter initial stationary phase and to stimulate a more rapid rate of growth than did equivalent amounts of saliva autoclaved in the medium (Fig. 2, *A*). In the case of the particular specimen illustrated in Figure 2, at a 5 per cent concentration, modification of total growth by this variation in technique was not apparent. However, at a 2.5 per cent concentration of the same saliva, greater total growth was observed when it had been separately sterilized (Fig. 2, *B*). The effect that autoclaving saliva in the basal medium had upon the total growth was obscured at the 5 per cent level by a combination of factors. Apparently, following autoclaving, the level of unaltered growth-promoting factor(s) in this saliva–basal medium mixture remained above that required for maximum growth. The difference in response, then, between saliva autoclaved separately and saliva autoclaved in the basal medium was obscured at the 5 per cent concentration by limiting factors in the basal medium and/or the physical properties of the culture medium.

Effect of the concentration at which saliva is autoclaved.—The reduced growth observed when saliva was diluted in the basal medium before autoclaving suggested that either dilution per se or inactivation of the nutrients in the saliva or basal medium might be responsible for the lessened response. In order to examine this first possibility, a comparison was made of the growth response to saliva, autoclaved as whole, undiluted saliva, and as a 10 per cent solution* in distilled water (or saline). It was subsequently shown, on the basis of the response per milliliter of saliva added to the basal medium, that the aliquots autoclaved before dilution produced a more rapid rate and a greater total growth than did equivalent amounts of the same salivary samples autoclaved as a 10 per cent aqueous solution (Fig. 3, *A*). In the case of the response to 0.2 ml. of saliva per tube of basal medium, illustrated by Figure 3, *A*, the aliquot autoclaved before dilution induced a shorter stationary phase of growth than did the one that was diluted in water and then autoclaved. However, this has been a less regular finding than the effect upon rate of growth and total growth.

Effect of pH at which saliva is autoclaved.—The salivary samples that were autoclaved separately had a pH value of 8.0+. This was in contrast with the pH of the buffered basal medium of 6.8, the pH at which the samples autoclaved in the medium were sterilized. The effect of this difference in pH was assessed by comparing the growth response to equivalent amounts of 2 aliquots of the same salivary samples, one aliquot autoclaved at a pH of 8.0+ and the other adjusted† to pH 6.5 before autoclaving. It was observed that the aliquots sterilized at the higher pH produced a more rapid growth and generally a greater total growth (Fig. 3, *B*).

Growth-promoting activity of salivary sediment and supernatant.—In an effort to characterize the stimulatory component(s) of saliva, its distribution between the simple centrifugal fractions was examined. Samples of autoclaved saliva were centrifuged in graduated centrifuge tubes for 20 minutes at 2,000 rpm. The centrifugal fractions were separated and reconstituted to original volume with sterile distilled water,

* The 1:10 dilution was chosen because it has been found to be convenient for accurately adding the graded volumes of saliva to the basal medium, over which a response proportional to concentration could be observed.

† The pH was adjusted by adding solid monobasic potassium phosphate to the saliva. The solid buffer was used to minimize dilution.

and the growth response to amounts of sediment and supernatant, equated on the basis of their relative levels in whole saliva, was determined. The results of these experiments showed that the sediments from autoclaved saliva induced little or no growth (Fig. 4).

This lack of response to the sediment from autoclaved saliva was shown to be due to the loss of growth factor from this fraction and not the result of inhibitor production or inhibitor disclosure by the loss or destruction of relatively more effective growth-promoting compounds. This was demonstrated by adding sediment from autoclaved saliva to supernatants and comparing the test micro-organism's response to the mixture with that of the supernatant alone. In none of five tests was the response to the mixture of sediment from autoclaved saliva and supernatant less than the response to equivalent amounts of the same supernatants.

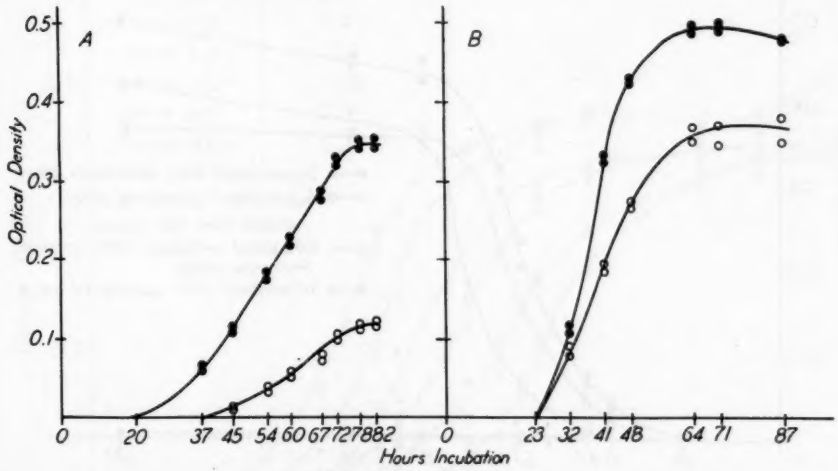

FIG. 3.—Growth response of an oral lactobacillus to 0.2-ml. aliquots of whole saliva (*A*), one autoclaved as whole undiluted saliva, ●—●, and one diluted 1:10 with distilled water before autoclaving, ○—○; and to 0.8-ml. aliquots of saliva sample (*B*), one autoclaved at pH 8, ●—●, and one at pH 6.5 (adjusted with KH_2PO_4), ○—○.

Subsequently it was found that the sediment effected a comparatively marked growth response if it was separated from raw saliva and resuspended to original volume in distilled water (or saline) before autoclaving (Fig. 4).

Separate sterilization of the centrifugal fractions also influenced the growth response elicited by the supernatants. The activity of this fraction was found to be consistently higher when it was separated from autoclaved saliva, in contrast to equivalent amounts of supernatant from an aliquot of the same salivary sample separated from its sediment prior to autoclaving (Fig. 4).

The lessened growth-promoting activity of the sediment from autoclaved saliva was not completely dependent on its being autoclaved as whole saliva. When the centrifugal fractions were autoclaved separately and then recombined for 18 hours, the activity of the sediment was also lost, whether the recombined sterile fractions were stored at 37° C., room temperature, 5° C., or were frozen. In contrast, aliquots of sediments

from the same salivary samples which had been separated from their supernatants and stored at freezing temperatures demonstrated activities comparable to sediment derived from the fresh raw saliva before autoclaving. In addition, it was noted that sediment removed from raw saliva that had been frozen for as short a period as 15 hours also demonstrated a loss of activity, in contrast to sediment removed from the same salivary samples shortly after collection. However, this loss was not so complete as that observed when comparable aliquots of the same particulate fractions were from autoclaved saliva or were recombined with autoclaved supernatants.

The decreased growth-promoting activity of the sediments from samples of saliva that had been frozen suggested that perhaps the freezing of saliva promoted changes in some component(s) that caused it to sediment with the particulate fraction and that

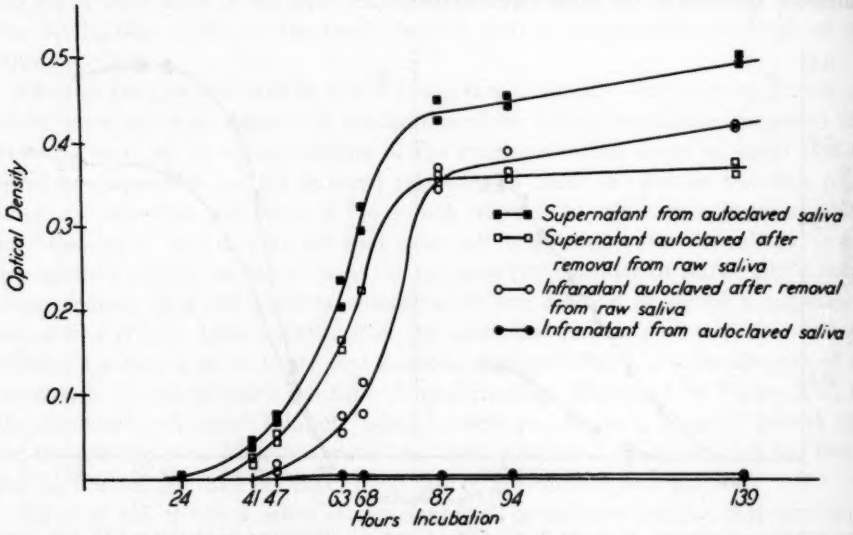

FIG. 4.—Growth response of an oral lactobacillus to 0.2 ml. of the centrifugal fractions of saliva separated before and after autoclaving. (Final volume 10 ml.)

this altered component was inhibitory. In fact, it was found that when supernatant fractions from fresh raw saliva were frozen (for 15 hours), a white flocculent material did precipitate when this fraction was thawed. No attempt was made to determine the exact nature of this material (probably, in part, some of the inorganic salts of saliva), but it was found that it neither inhibited nor enhanced the growth response of the test micro-organism to the infranatant. Neither did the loss of this material from the supernatant result in any marked change in the supernatant's stimulatory activity.

It might be expected that a study of the centrifugal fractions of saliva would lead to results that would show which fraction induces the most rapid and greatest growth of the test lactobacillus. However, this was not the case, since the relative activity of supernatant and sediment was found to vary from one sample of saliva to another.

Effect of pH on the stimulatory activity of salivary sediment and supernatant.—To determine whether the decreased stimulatory activity of the sediments recovered from autoclaved saliva was, in part, the result of the high pH at which they were sterilized,

the pH of aliquots of resuspended sediment was adjusted with phosphate buffer and hydrochloric acid, in one pH unit increments, between pH 5 and pH 9, before they were autoclaved. A test of the growth response to equivalent amounts of these aliquots showed an inverse relationship to the pH at which they were autoclaved (Fig. 5). The decreased growth response was again demonstrated to be a reflection of growth-factor destruction, and the addition of an aliquot of salivary sediment, autoclaved at pH 9, that would not induce growth of the test micro-organism in the basal medium, to another sterilized at pH 5 (highly stimulatory), never resulted in a decreased response over that seen with the latter alone.

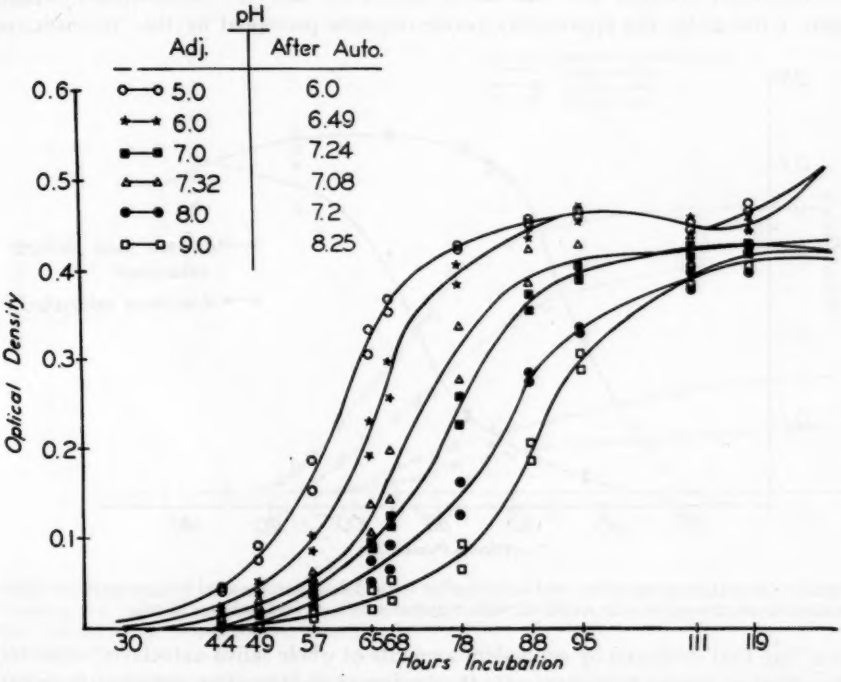

FIG. 5.—Growth response of an oral lactobacillus to salivary infranatant, demonstrating the effect of pH at which the sample was autoclaved on response. 0.02 ml resuspended infranatant/ml medium.

However, the effect that autoclaving sediment as whole saliva has upon its stimulatory activity cannot be entirely explained by the pH effect. Raw sediment resuspended in distilled water (pH 6.9) that was sterilized with ethylene oxide (pH 7.6 following treatment) showed considerably greater growth-inducing potential than did an autoclaved aliquot from the same sample that had been sterilized at a lower pH (6.9 following autoclaving) (Fig. 6).

Autoclaving the supernatant of saliva at varying hydrogen-ion concentrations was also observed to influence the growth response elicited by this fraction, although in a manner opposite to that seen with the infranatant. The growth-stimulatory effect of the supernatant decreased when the pH at which it was autoclaved was reduced from 9 to 5. This effect was not so pronounced as in the case of the sediment; a considerably

greater difference in pH (3–4 units) was required to produce a small, but consistent and demonstrable, effect.

Stimulatory effect of "reconstituted" saliva.—Following the observation that the activity of salivary sediment can be demonstrated when it is autoclaved separately from the supernatant, it was of interest to determine whether this stimulatory effect of the sediment would be additive to that produced by the supernatant. Using samples of saliva from individuals who had consistently provided specimens of low stimulatory activity (to preclude levels of response that would be limited by the culture system), a comparison was made of the growth-promoting activities of whole saliva in which the centrifugal fraction was autoclaved separately and of "reconstituted"* saliva. Figure 7 illustrates the appreciably better response prompted by the "reconstituted"

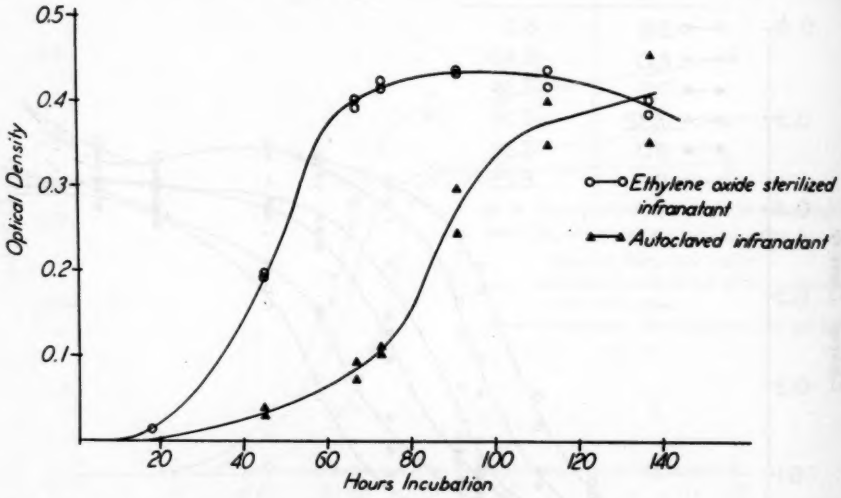

FIG. 6.—Growth response of an oral lactobacillus to 0.6 ml. of resuspended infranatant; one aliquot was autoclaved, the other was sterilized with ethylene oxide. (Final volume 10 ml.)

saliva than that produced by equivalent amounts of whole saliva autoclaved separately.

In addition, Figure 7 demonstrates the consistent finding of an apparent synergistic effect of the two centrifugal fractions autoclaved in combination. The response to saliva autoclaved as whole saliva was greater than can be explained by a simple additive effect of the two separately sterilized centrifugal fractions. The "reconstituted" saliva demonstrated the similar, greater-than-additive effect, plus the additional component of growth response characteristic of the separately autoclaved sediment. In the case of autoclaved whole saliva, the increased response may be, in part, the result of the enhanced activity shown by supernatant fractions autoclaved with the infranatant.

DISCUSSION

If most of the steps required to perform a microbiological assay can be accomplished before the system is sterilized, the opportunity for chance contamination is

* Reconstituted saliva was prepared by adding the separately autoclaved centrifugal fractions to the sterile basal medium in amounts that re-established the normal proportions in whole saliva.

considerably reduced. In addition, the complication of carrying out a more completely aseptic technique is minimized if a greater number of steps can be performed before asepsis is established. However, there remains the possibility that autoclaving a chemically complex material like saliva in the relatively complete synthetic medium might result in heat-catalyzed changes. These changes might, in turn, affect the response of the test micro-organism as the result of alterations in essential nutriments, such as that seen when glucose is autoclaved in the basal medium.[7] Also, heat and a low pH have been shown to decrease the reducing coefficient of saliva,[11] and a low oxidation-reduction potential favors growth initiation by the microaerophilic lactic acid bacteria.[12]

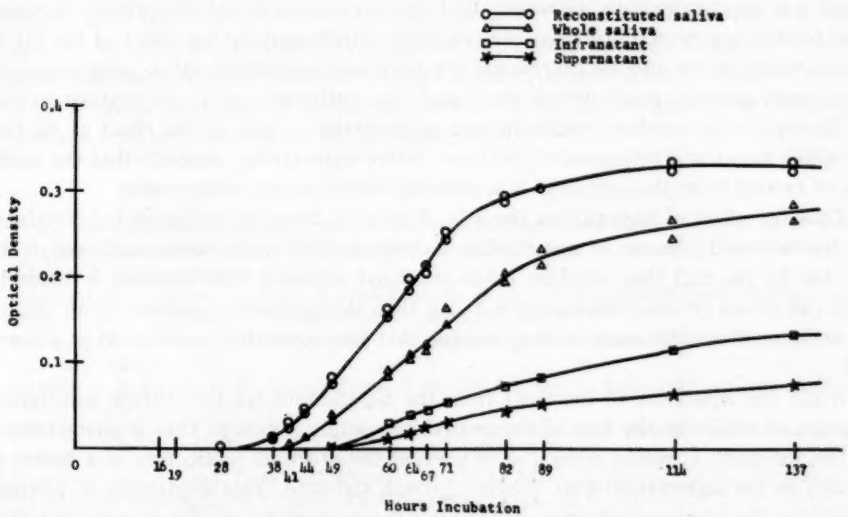

FIG. 7.—Response of an oral lactobacillus to 0.6 ml. of whole saliva and equivalent volumes of "reconstituted" saliva, supernatant, and infranatant from the same salivary specimen. Fractionated prior to autoclaving. (Final volume 10 ml.)

The observations presented here indicate that a certain sequence of steps in the assay procedure should be observed if reproducible and reasonably valid assessments of saliva's growth-promoting potential, as it likely obtains in the oral cavity, are to be achieved. Undoubtedly, autoclaving produces marked changes in the composition of saliva, and autoclaved saliva may very well not even approximate the basic medium of the oral protista. The above data demonstrate a number of such changes that take place during autoclaving. It is also apparent from the results that these changes can be eliminated by controlling certain presumptive reactions that apparently alter the composition of saliva, as indicated by their effects upon the growth of the test micro-organism.

The effect that diluting saliva in water or basal medium before autoclaving has upon its growth-promoting activity appears to be due principally to an alteration of the sediment. This impression derives from the finding that diluting a sample of saliva (in water) before autoclaving did not result in any demonstrable effect upon its stimu-

latory activity if the particular sample of saliva contained a particulate fraction that did not, itself, show appreciable activity when removed from the fresh, raw salivary specimen before autoclaving. In addition, it has been observed that the response to equivalent amounts of aliquots from the same supernatant varies little, whether the aliquot is diluted 1:10 prior to autoclaving or is sterilized undiluted. Also, autoclaving a 1:10 dilution of a relatively stimulatory resuspended* sediment before addition to the basal medium results in an almost complete loss of its stimulatory activity.

The loss of growth-promoting activity from the sediment, observed when it is autoclaved or stored as whole saliva, is due to a combination of circumstances. One such factor, and one that also contributes to the coincident gain in stimulatory activity of the supernatant, is the apparent direct dissolution of component(s) from the sediment into the supernatant. However, that this occurrence is not the primary explanation for the loss of activity from the sediment is indicated by the effect of the pH at which whole saliva and its centrifugal fractions are autoclaved. Also, comparisons of the growth-promoting activity of whole and "reconstituted" saliva, in contrast to that of the separately sterilized sediment and supernatant, as well as the effect of dilution on whole saliva and resuspended sediment before autoclaving, suggests that the leaching of factors from the sediment is apparently of secondary consequence.

That the effect of high pH on the loss of activity from the sediment is potentiated by the heat and pressure of autoclaving, as well as other undisclosed conditions, is indicated by the fact that ethylene oxide–sterilized sediment that develops a relatively high pH shows greater stimulatory activity than do equivalent amounts of an aliquot of sediment from the same salivary sample that was separately autoclaved at a lower pH.

While the separation of sediment from the supernatant fraction before autoclaving appears to minimize the loss of the growth-promoting potential that is characteristic of the sediment, it would seem also to prevent the artificial production of a factor or factors in the supernatant that enhance growth response. This impression is substantiated by the observed increase in the stimulatory activity of the supernatant that parallels the increase in pH at which it is autoclaved, as well as the greater-than-additive response of separately autoclaved whole saliva over that of its separately autoclaved sediment fractions and supernatant. This relatively increased stimulatory activity of supernatant and the concomitant decrease in the activity of the sediment can, in a large part, be reasonably explained on the basis of an alteration in the oxidation-reduction potentials of the two fractions. This impression takes credence from the findings that the reducing activity of saliva is primarily associated with the sediment and that its reducing intensity is maximum at a pH of 8 and is destroyed by heating.[11] This explanation would fit the observations; a more oxidized sediment from autoclaved saliva that would be less stimulatory for the microerophilic test micro-organism and a more reduced supernatant fraction would be more stimulatory. The same reasoning would apply to the effect of pH on whole saliva, since the reduction of components in the supernatant by the sediment would be facilitated at the higher pH. Also, the effect of dilution on whole saliva and sediment can be related to the effect on the oxidation-reduction potentials of the fractions; concentration would affect the

* Initially resuspended in distilled water to a volume equivalent to that of the whole salivary aliquot from which the sediment was derived.

reducing activity of the sediment. This explanation, however, would depend on the assumption that the Eo of the oxidation-reduction systems in the supernatant is greater than those in the sediment and that the reduced form of components in the supernatant has a considerably greater growth-promoting effect on the test bacteria than do the reduced components of the sediment.

SUMMARY

Examination of the technique for the microbiological assay of saliva using an oral strain of *L. casei* as the test micro-organism revealed that if a more valid assay is to be achieved, the centrifugal fractions of saliva should be promptly separated. The separate fractions can then be autoclaved, appropriately diluted, and added aseptically to the sterile basal medium. If it becomes necessary to store the samples before they can be assayed, the separate fractions can be placed in the deep freeze. The data presented illustrate that this particular sequence of steps apparently minimizes the destruction of stimulatory compounds present in the infranatant, as well as precluding the formation of probable artificial metabolites in the supernatant.

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Inhibition of Bacterial Growth in Human Saliva by Vitamins B_1, B_2, and B_6

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Media containing yeast or yeast extracts, both rich in vitamins of the B complex, have been used for a long time in bacteriological cultures. Some components of the vitamin B complex (riboflavin, pyridoxine, inositol, and biotin) may be essential growth factors for some bacteria and Protozoa.[1]

Small amounts of thiamin or pyridoxine show a partial inhibitory effect, and riboflavin produces complete inhibition of human salivary amylase activity in vitro. It has also been observed that albino rats kept on a caries-producing diet failed to develop caries if thiamin, riboflavin, and pyridoxine were administered simultaneously.[2, 3] In other experiments it has been noticed that human saliva incubated with thiamin, riboflavin, and pyridoxine did not develop odor, while controls incubated without these vitamins were foul-smelling. Since this observation suggested decreased bacterial activity in the presence of thiamin, riboflavin, and pyridoxine, the following experiments were carried out.

EFFECT OF B VITAMINS ON AEROBIC BACTERIAL GROWTH

Experiment I: Materials and methods.—Three ml. of beef broth were pipetted into each of 11 sterile test tubes. Three ml. of a sterile aqueous solution containing 0.6 mg. of thiamin hydrochloride (vitamin B_1), 1.2 mg. of riboflavin (vitamin B_2), and 0.6 mg. of pyridoxine hydrochloride (vitamin B_6) were then added to test tube 1. After thorough shaking, 3 ml. of the contents of test tube 1 were pipetted into tube 2. Serial dilutions (1:2 to 1:1024) of the vitamin solution were then obtained in 3 ml. of broth. One tube contained only broth (pH 7.6) and served as control. The pH of test tube 1 was 5.6; with dilution of the vitamin solution, the pH rose correspondingly. Finally, 0.1 ml. of normal human saliva (collected and handled under sterile conditions) was pipetted to all test tubes. These tubes were incubated aerobically at 37° C. and examined after 24 hours.

Results.—As demonstrated in Figure 1, the control test tube (*C*) showed strong turbidity of the medium, whereas the broth in test tubes 1, 2, and 3 was perfectly clear. (The sediment in them consisted of food particles, desquamated oral epithelial cells, and salivary mucus.) Tubes 4, 5, and 6 showed increasing turbidity, whereas tubes 7, 8, 9, and 10 demonstrated dense turbidity. Further incubation (up to 72 hours) did not alter these results.

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The bacteria were identified as *Staphylococcus aureus, S. albus,* non-hemolytic group *A Streptococcus,* and *Sarcina lutea.*

Experiment II: Materials and methods.—The procedure was similar to Experiment I, but the final pH was adjusted to 7.0 with 0.1 M $NaHCO_3$ in every tube.

Results.—The pattern of bacterial growth was similar to that in previous experiments, but was less abundant.

Experiment III: Materials and methods.—Melted agar was poured into Petri dishes and cooled to 48° C. Then 3 ml. of the vitamin solution used in Experiments I and II was added to Plate 1. A dilution series was made in the same way as in Experiments I and II. Afterward 0.1 ml. of normal human saliva was added to the medium and incubated for 24 hours aerobically at 37° C.

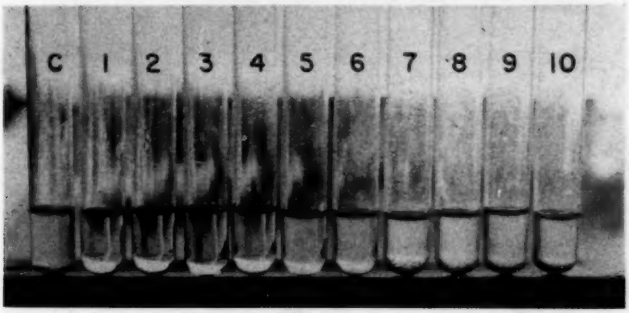

FIG. 1.—Dilution series of the B vitamin mixture added to saliva and beef broth. Notice the clearness of the medium in test tubes 1, 2, and 3, in which the vitamin solutions were less diluted. The medium in tubes 4–10 and in the control (*C*) is turbid, because of bacterial growth.

Results.—Abundant bacterial growth was observed in the control (Fig. 2, *A*), and in the Petri dishes with higher dilutions of the vitamin mixture (plates 4–10). Dishes containing 1:2, 1:4, and 1:8 dilutions of the vitamin solution (plates 1, 2, and 3) had significantly less bacterial growth than the control (Fig. 2, *B*).

EFFECT OF B VITAMINS ON ANAEROBIC BACTERIAL GROWTH

Experiment IV: Materials and Methods.— To each of four sterile tubes with Tarozzi's medium,* 1 ml. of normal human saliva was pipetted. To tube 1 an aqueous solution of thiamin hydrochloride was added, the final concentration being 0.04 per cent. Solutions of riboflavin and pyridoxine hydrochloride were added to tubes 2 and 3, respectively, with the same final concentration. Tube 4 served as control. The tubes were incubated anaerobically for 48 hours at 37° C.

Results.—No bacterial growth was observed in the tubes containing thiamin hydrochloride or riboflavin; minimal growth was seen in the presence of pyridoxine hydrochloride. The controls showed abundant bacterial growth.

Experiment V: Materials and methods.—The method was the same as in Experiment IV, but incubation was carried out at 20° C.

Results.—Identical with those of the previous experimental series.

*Tarozzi's medium: broth made of and containing small cubes of hog liver, sealed anaerobically; pH 7.4.

The bacteria in these experiments were identified as anaerobic streptococci, *Staphylococcus aureus, S. albus,* and Sarcinae.

EFFECT OF TEMPERATURE ON BACTERIAL GROWTH IN THE PRESENCE OF B VITAMINS

Experiment VI: Materials and methods.—In this series normal human saliva was incubated with single vitamin solutions (of thiamin hydrochloride, riboflavin, and pyridoxine hydrochloride, respectively). The final concentration of the vitamin was 0.04 per cent in the corresponding tube. One series was incubated at 20° C., the other at 37° C. After 5 days of aerobic incubation the contents of each tube were plated on blood agar, using half of each plate for control (saliva incubated alone). The agar plates were incubated aerobically for 24 hours at 37° C.

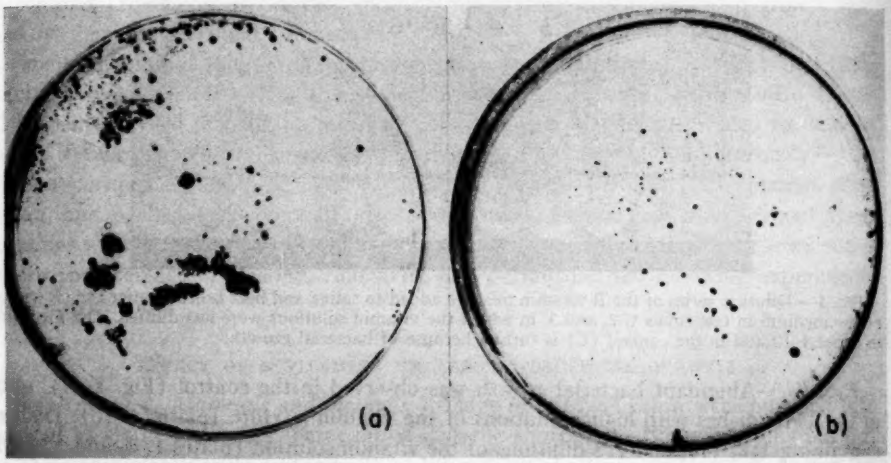

FIG. 2.—Agar plates inoculated with bacteria from saliva. The bacterial colonies are black on the photograph. Decreased bacterial growth could be observed with B vitamins added to the medium (*B*) as compared with the control (*A*).

Results.—There was no bacterial growth after incubation (both at 20° and at 37° C.) of the saliva with thiamin hydrochloride (Fig. 3) or riboflavin; a striking decrease in bacterial growth was found with pyridoxine hydrochloride (Fig. 4), as compared with controls.

EFFECT OF pH ON BACTERIAL GROWTH IN THE PRESENCE OF B VITAMINS

Experiment VII: Materials and methods.—One ml. of normal human saliva was pipetted into each of three sterile test tubes. To test tube 1, 1 ml. of a vitamin solution (aqueous solution containing 0.2 mg. of thiamin hydrochloride, 0.4 mg. of riboflavin, and 0.2 mg. of pyridoxine hydrochloride) was added. The final pH of this mixture was 4.2. Test tube 2 contained saliva only (pH 7.1). The saliva in test tube 3 was brought to pH 4.2 by the addition of HCl. The volumes of the latter two tubes were adjusted to 2 ml. with physiologic saline solution. Thus a 1:2 dilution of the saliva and presumably of its bacterial content was achieved in all three tubes. After

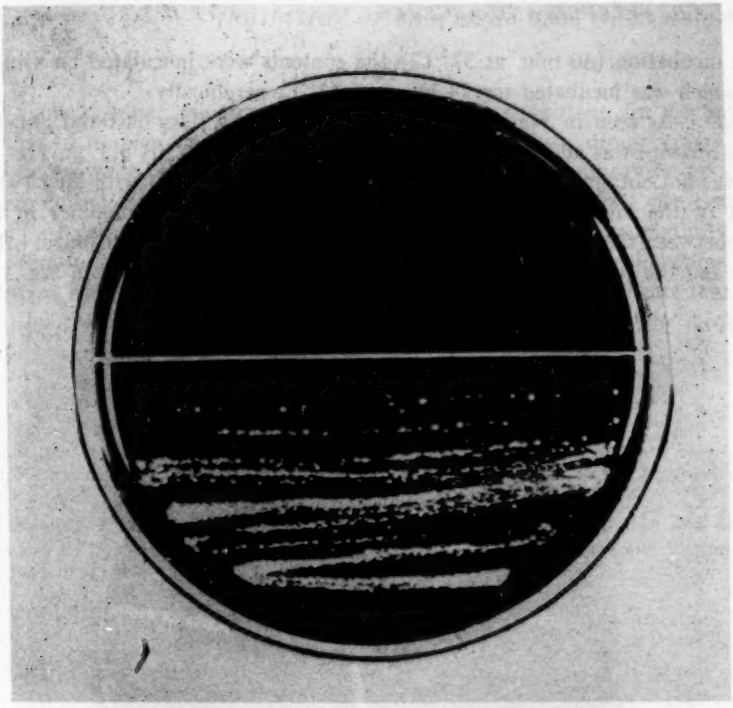

FIG. 3.—No bacterial growth was seen with thiamin hydrochloride added to the saliva before inoculation (*upper half of the agar plate*). Many bacterial colonies grew from the saliva without added thiamin hydrochloride (*lower half of the plate*).

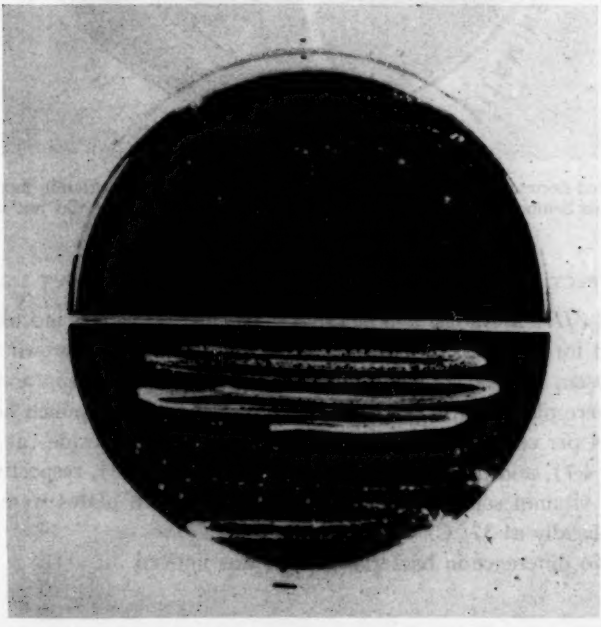

FIG. 4.—Markedly decreased bacterial growth was observed with pyridoxine hydrochloride added to the saliva prior to inoculation (*upper half of the agar plate*). The control (*lower half of the plate*) showed abundant bacterial growth.

a short incubation (30 min. at 37° C.) the contents were inoculated on a blood agar plate, which was incubated for 24 hours at 37° C. aerobically.

Results.—As seen in Figure 5, there was significantly less bacterial growth if the vitamin mixture was added to the saliva (labeled "Vitamins pH 4.2" in Fig. 5). This result was not obtained by bringing the pH of the saliva to 4.2 with HCl (which was the pH of the vitamin mixture). Actually, there was little difference in bacterial growth between the inoculum from the saliva at pH 4.2 (labeled "Control pH 4.2" in Fig. 5) and the control saliva at pH 7.1 (labeled "Control pH 7.1" in Fig. 5).

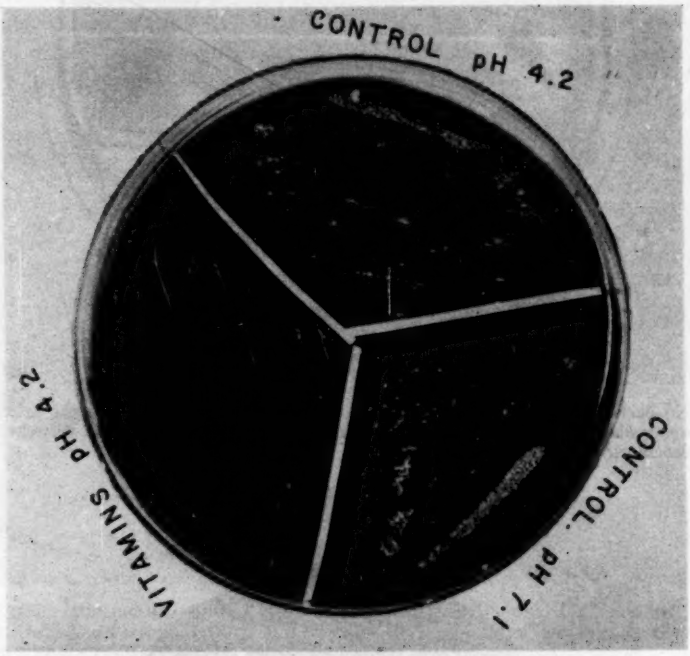

FIG. 5.—Marked decrease in bacterial growth was noticed with the B vitamin mixture added to the medium. Controls demonstrate that change in pH of the medium alone did not influence bacterial growth.

EFFECT OF B VITAMINS ON BACTERIAL GROWTH WITHOUT SALIVA

Experiment VIII: Materials and methods.—Normal saliva was inoculated into broth and incubated for 24 hours aerobically at 37° C. The bacteria grown were identified as *Staphylococcus aureus, S. albus,* non-hemolytic group *A Streptococcus,* and Sarcinae. They were plated on blood agar, which had three small punch holes containing 0.1 ml. of 0.04 per cent aqueous solution of thiamin hydrochloride (at pH 2.4), riboflavin (at pH 4.7), and pyridoxine hydrochloride (at pH 6.8), respectively. A control plate without vitamin solutions was also inoculated. Both plates were incubated for 24 hours aerobically at 37° C.

Results.—No difference in bacterial growth was noticed.

SUMMARY

Normal human saliva was incubated under various conditions for bacteriologic study. Strains of *Staphylococcus aureus, S. albus,* non-hemolytic group *A Streptococcus,* and Sarcinae were identified in it. These bacteria showed significantly less growth if they were cultured with 0.02 per cent thiamin hydrochloride, 0.04 per cent riboflavin, and 0.02 per cent pyridoxine hydrochloride (singly or in their mixture) added to saliva prior to incubation. This result was not due to the simple lowering of pH which occurred with the addition of vitamin solutions. The findings indicate that saliva is essential for the observed bacteriostatic action of these B vitamins. The mechanism of action of this effect is under investigation.

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Immunochemical Studies of Human Saliva. I. The Demonstration of Serum Proteins in Whole and Parotid Saliva

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Human saliva is known to contain a number of enzymes and other protein constituents, but only one of these—salivary amylase—has been adequately characterized.[1, 2] Electrophoretic data have indicated the presence of at least seven components, whose identity, deduced from such studies, must be considered largely conjectural.[3–11] Immunochemical agar diffusion techniques[12, 13] appear uniquely suitable to the problem of enumerating and identifying the antigenic substances in saliva. The results below are illustrative of those obtained by employing a plate diffusion technique and agar immunoelectrophoresis. By these means, several proteins have been distinguished, some of which have been identified as being serologically unaltered serum proteins.

EXPERIMENTAL METHODS

Preparation of antisera.—Paraffin-stimulated whole saliva was collected from 35 persons and pooled. It was then clarified by centrifugation at 4° C. at 1060 × G for 1 hour. The supernatant was decanted, filtered through a coarse sintered-glass funnel to remove the paraffin, and used to prepare an alum-precipitated antigen by adding 5 ml. of 1 per cent potassium aluminum sulfate per 100 ml. of filtrate and adjusting the pH to give maximum flocculation (approximately pH 6.5).[14] A portion of the filtered pool was stored at —20° C. and used to prepare fresh antigen in like manner as required during the course of immunization. Rabbits were immunized by intravenous injection of the antigen four times weekly for 4 weeks, each receiving a total of 20 ml., and were bled 10 days after the last injection. After a 2-month interval, the animals were given a second similar course of immunizing injections with an antigen prepared from the same stored saliva. Rabbit antisera were also prepared by immunization with antigen plus adjuvant, employing Freund's mixture[15] or an Arlacel-Bayol F emulsion.[16] The pooled saliva was concentrated ten times by pervaporation at 4° C. for use in these antigens. Injection was made intracutaneously or subcutaneously, and blood specimens were collected 9–11 days subsequently. The animals were reimmunized at approximately bimonthly intervals.

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Antiserum to human serum and human serum fractions were obtained from animals immunized with adjuvant vaccines made by using Bayol F and Arlacel. The schedule of bleedings and immunizations was similar to that employed with saliva.

Agar diffusion methods.—Agar plates for diffusion were prepared by the Ouchterlony method as modified by Halbert, Swick, and Sonn.[17] These were kept in the refrigerator and observed at frequent intervals for periods up to 1 month before being discarded.

For immunoelectrophoresis, a modification of the slide microtechnique of Scheidegger[18] was employed. The electrophoretic cell was constructed from a compartmented plastic drawer-divider and utilized platinum electrodes. The agar was supported on glass slides, either 25 × 100 mm. or 60 × 100 mm. Sufficient 2 per cent buffered agar was distributed over the slide to provide an even film (2.5 or 6 ml., respectively, for the small or large slides). Filter-paper strips, 5 mm. narrower than the slide, were moistened in buffer and placed on the agar at the ends of the slide and served to connect them to the electrode cells. In early experiments a cutter, fabricated according to the directions of Scheidegger,[18] was employed; it was later found more expedient to cut the antiserum wells by hand after electrophoresis. Voltage was adjusted to give a current of 3 ma. for each narrow slide or 6 ma. for each wide slide (90–130 volts). A glass plate was used to seal the apparatus during the approximately 2 hours of electrophoresis. After this, the paper electrodes were removed, the troughs cut as required, and the antiserum added. The slides were incubated for at least 1 week in the refrigerator in sealed plastic boxes.

The antigens employed in testing the antisera included whole saliva, parotid saliva collected by means of a modified Curby cup,[18] sublingual-submaxillary saliva obtained by using a collector as described by Schneyer,[20] human serum, and purified serum proteins.*

RESULTS

The serums obtained from animals immunized with the alum-precipitated antigen gave multiple bands in agar diffusion plates when tested with saliva and other antigens. Examples of these are shown in the photographs and diagrams in Figure 1. It will be seen from Figure 1, *A*, that three antigens were disclosed in parotid saliva, at least one of which could be identified also in whole saliva. One of the components in whole saliva was absent both from serum and from parotid saliva and might represent either a bacterial antigen or a distinctive constituent of the submaxillary-sublingual secretion. At least six bands were present in the agar between the well containing antiserum and that containing undiluted human serum. For the most part, the reacting serum proteins did not appear to be present in saliva in a concentration sufficient to be directly detected by agar diffusion tests using this serum. The antiserum whose reaction pattern is shown in Figure 1, *B*, had quite a different spectrum of antibodies than did the preceding one. At least five bands were present in the agar between the well containing antiserum and that containing saliva. Four components were detected in parotid saliva. Three of the bands produced by whole saliva were seen to be

* The parotid cups were loaned by Dr. I. Zipkin, National Institute of Dental Research. Dr. B. F. Erlanger, Columbia University, and Dr. Roy K. Brown, New York State Department of Health, supplied the purified serum proteins.

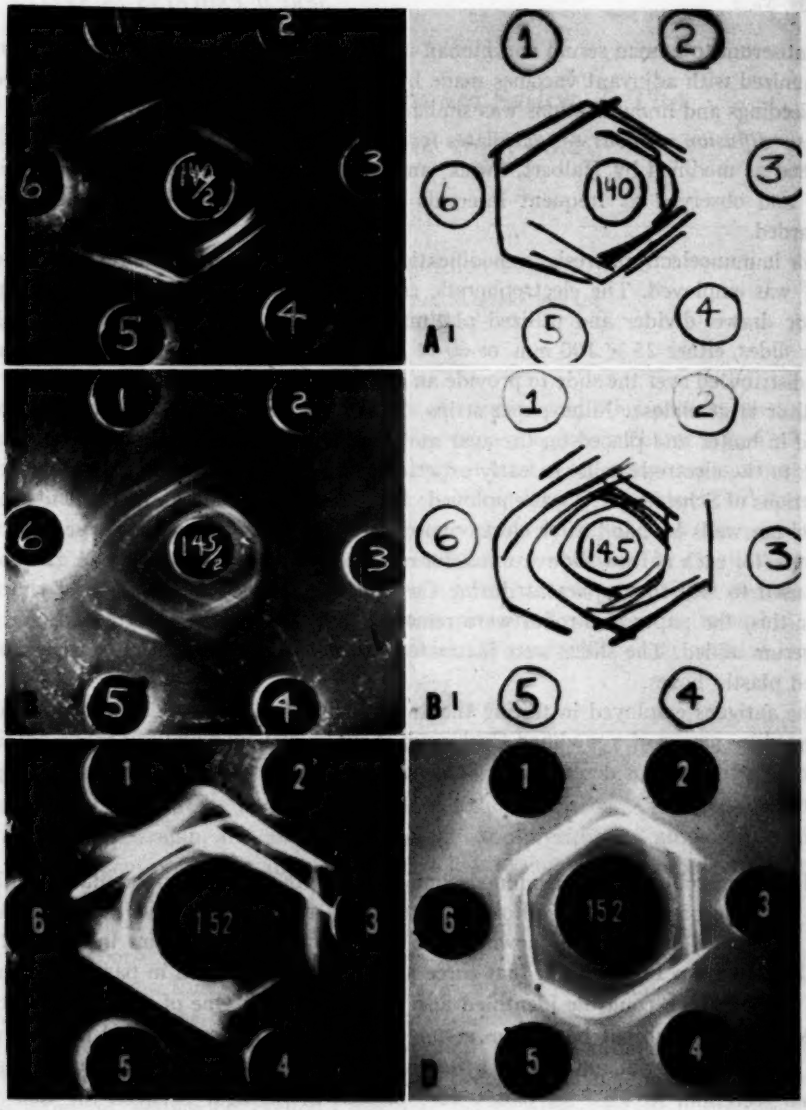

FIG. 1.—Photographs and diagrams of agar diffusion plates. In each instance, an antisaliva serum was placed in the center well and the antigens in the peripheral wells. *A* and *A'*—the center well contained serum 140/2; the peripheral wells contained the following: *1*, whole human saliva; *2*, human serum; *3*, whole human saliva, diluted 1/10 with 0.9 per cent saline; *4*, human serum, diluted 1/10 with 0.9 per cent saline; *5*, human parotid saliva; *6*, human parotid saliva, diluted 1/10 with 0.9 per cent saline. *B* and *B'*—the center well contained serum 145/2; the peripheral wells contained the same antigens as in the preceding instances. *C*—the center well contained serum 152/4–1; the peripheral wells contained the following: *1*, whole human saliva; *2*, human parotid saliva; *3*, serum fraction II-1, 2 (human γ-globulin); *4*, β-globulin + serum albumin (human); *5*, human serum, diluted 1/10 with 0.9 per cent saline; *6*, serum fraction V (human serum albumin). *D*—the center well contained serum 152/4–2; the peripheral wells contained the following: *1*, whole human saliva; *2*, human submaxillary saliva; *3*, human serum, diluted 1/80 with 0.9 per cent saline; *4*, whole human saliva; *5*, human submaxillary saliva; *6*, human parotid saliva.

due to antigens in parotid saliva, since the respective bands joined.[21] In addition, a number of serum proteins were distinguished; one appeared to be identical with an antigen in parotid saliva and one, quite probably the same, was also detected in whole saliva.

With stronger antiserums, obtained from animals immunized by adjuvant vaccines, serum proteins were directly demonstrated in saliva. Figure 1, *C*, for example, shows an agar diffusion plate in which a serum from a rabbit which received four courses was tested with various antigens. The number of reacting components in saliva and serum was so great as to render resolution of the bands difficult. It may be seen, however, that the band with γ-globulin joined with one of a group formed with parotid saliva. Similarly, a continuous band due to serum albumin was found between the antiserum well and the wells containing whole saliva, serum albumin, whole serum, and a mixture of β-globulin and albumin. Figure 1, *D*, shows the reaction of another such antiserum and demonstrates the serological identity of components in submaxillary and parotid secretions and the presence of serum proteins in both.

The results obtained with the microtechnique of immunoelectrophoresis employed in these studies were found to be very much dependent on the concentration of the antigen. It was essential to concentrate the saliva prior to testing because of its low protein content. This effect was evident in the reactions of whole saliva (Fig. 2, *A*) and parotid saliva (Fig. 2, *B*). Both the number and the intensity of the bands increased as more concentrated antigen was employed.

The slide illustrated in Figure 2, *C*, shows a comparison of the immunoelectrophoretic pattern of ten times concentrated whole saliva and parotid saliva. Human serum was placed in wells *1* and *4*, whole saliva in well *2*, and parotid saliva in well *3*. After electrophoresis, an antisaliva serum was placed in the center trough and antiserum serum in the two lateral troughs. The photograph was taken after 72 hours' incubation at 4° C. The various components in the salivas may be provisionally identified by comparing their position with those of the serum proteins. In this sample of whole saliva, two proteins with the mobility of serum albumin and three which migrated as γ-globulin were detected with the antisaliva serum; the antiserum serum disclosed albumin and γ-globulin. Although the parotid saliva showed corresponding reactions with both serums, differences were seen in the density and in the position of the bands. A curious finding was the presence of a component which was not clearly resolved electrophoretically. This is illustrated in Figure 2, *C*, and shown more clearly in Figure 2, *D*. Two demilunes of precipitate are seen in the latter, indicating separation of an antigen into two portions, one of which has migrated to the anode and the other to the cathode. Since the bands join, without a spur, it is presumed that the proteins were immunologically similar. Bands with this appearance have been frequently observed in different salivas using various antiserums and do not appear to be artifactual.

More rigorous proof of the identity of the proteins may be obtained by using specific antiserums. Figure 2, *E*, shows the result obtained when antisaliva serum, antialbumin serum, and antiγ-globulin serum were used to develop the pattern. Human serum was placed in wells *1* and *4* and whole saliva in wells *2* and *3*. The correspondence of the albumin bands in serum and saliva was apparent, as was the presence of a band in whole saliva in the same position as the γ-globulin band of serum. Tests with

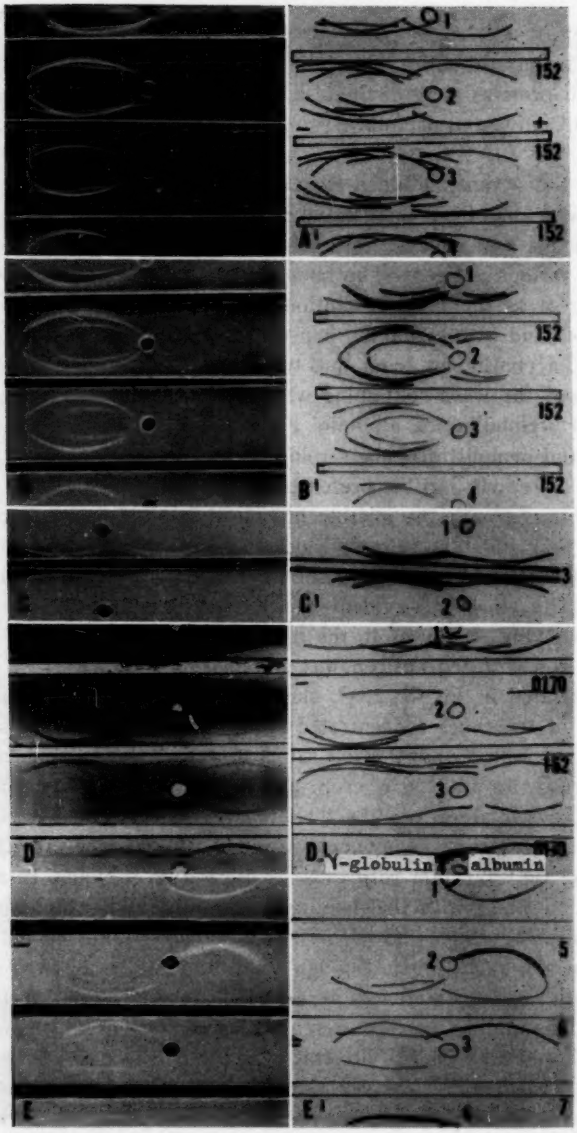

FIG. 2.—Photographs and diagrams of immunoelectrophoresis slides. The illustrations are oriented so that the positive (+) electrode is to the right, and the negative (—) electrode to the left. *A* and *A'*—the wells contained whole saliva as follows: *1*, ten times concentrated; *2*, five times concentrated; *3*, two times concentrated; *4*, unconcentrated. All troughs contained serum 152 antisaliva), *B* and *B'*—the wells contained parotid saliva concentrated as in *A* and *A'*; the troughs contained serum 152. *C* and *C'*—wells *1* and *4* contained human serum; well *2* contained whole saliva, ten times concentrated; well *3* contained parotid saliva, ten time concentrated. The center trough contained serum 152 (antisaliva); the lateral troughs contained serum 0170 (antiserum to human serum). *D* and *D'*—wells *1* and *2* contained ten times concentrated whole saliva. The trough (*3*) contained serum E-15-11/2 (antisaliva). *E* and *E'*—wells *1* and *4* contained human serum; wells *2* and *3* contained ten times concentrated whole saliva. The upper trough (*5*) contained serum 792 (antihuman serum albumin). The center trough (*6*) contained serum E-15-11/2 (antisaliva). The lower trough (*7*) contained serum 148 (antihuman γ-globulin).

purified salivary amylase have indicated that the heavy band formed with antisaliva serum in the globulin region was not a serum protein but was salivary amylase.

DISCUSSION

The extremely complex character of saliva is readily evident from the foregoing observations. Two distinct groups of antigens seem to be present. Some are serum proteins, and others are distinctive intrinsic salivary substances. The identification of γ-globulin may explain many of the bactericidal effects produced by saliva,[22] since this globulin is the antibody containing fraction of serum.

Although "quantitative" modifications[23, 24] of gel diffusion techniques have been suggested, no attempt has been made to use them here. No relation can be presumed between the intensity of reaction of an antigen with its antibody in an antiserum containing many antibodies and the concentration of the antigen. The components found are those to which one has obtained antibodies. The presumption that the most readily detected substances necessarily represent the principal components is not valid. Another point to be considered is the possible presence in saliva of substances which are not antigenic for rabbits, such as proteins of low molecular weight or polysaccharides, such as blood-group substances.

The precision of qualitative analysis of mixtures using immunochemical gel diffusion techniques can best be appreciated by comparing the results with those derived from electrophoretic studies. The latter method has been used by a number of investigators, and the identity of individual components inferred by comparing mobilities with known proteins, or else merely presumed on the basis of a rough analogy with serum. On this basis, for example, some workers have claimed that serum albumin is in parotid saliva,[5, 6, 8, 9, 11] whereas others have failed to find it.[7, 10] With specific antialbumin serum, albumin has been detected regularly. It cannot be stated, however, that this or other components found in these studies with antiserums correspond to those reported by other workers using different techniques. Experiments designed to elucidate these points have been begun and are continuing.[25]

In addition, it must be noted that, although strong evidence has been presented here for the identity of some serum and salivary proteins, it is still essential that the individual substances be isolated from saliva, characterized, and directly compared with the corresponding materials in serum in order to establish their identity absolutely.

SUMMARY

Rabbits were immunized with pooled whole human saliva. Agar diffusion studies employing the resultant antisera and various antigens showed the extremely complex nature of saliva. The experimental findings indicated that it contained unaltered serum proteins as well as distinct salivary antigens.

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Mechanical Failure of Amalgam Restorations with Zinc Phosphate and Zinc Oxide/Eugenol Cement Bases

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As a guide to operative practice it was desirable to obtain pertinent information on the mechanical performance of base materials and Class I amalgam restorations. Specifically, the question was: Under what circumstances, if any, can the interaction product of zinc oxide and eugenol be substituted for a zinc phosphate cement base?

Our investigations, then, have been concerned with the mechanical failure of two different types of Class I cavity preparations that have been filled with amalgam with or without these two kinds of underbase materials. Our tests have been designed to simulate actual mechanical forces of mastication, insofar as practicable, in connection with laboratory tests.

MATERIALS AND METHODS

In order to measure the comparative ease of failure on laboratory models, it was assumed that a fair evaluation could be obtained by measuring forces applied to the occlusal surface of the amalgam filling by means of an implement which could represent either an opposing cusp or a foreign object which was resisting the forces of mastication.

Accordingly, the test loads were applied to the amalgam fillings by means of a ⅛-inch steel ball that could be accurately seated in two intersecting channels (central fossa) on the occlusal surface. The condensation of the amalgam, then, was carried out not with the intent of producing lifelike topography (anatomy) but with the intent of obtaining a uniform thickness of amalgam and uniformity of the depth and definition of the channels in which the pressure was to be applied.

The amalgam was condensed in several layers, the final surface anatomy being produced by a die‡ fastened to a hand press (Fig. 1). This die had to be cleaned to remove excess amalgam after each use and probably suffered from abrasion during the testing program. A metal die might have been better, but it would have had to be fabricated from a metal which would not interact with the amalgam; this restriction eliminated several common metals that could be cast by usual laboratory methods.

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‡ PermaRock, Permadent Products, 1780 Broadway, New York 19, N.Y.

During the condensation of amalgam, as well as during the application of destructive forces, the plastic teeth were held in a jig (Fig. 5) which accurately fitted the teeth from the apex to within about 1 mm. of the crown. This jig was prepared by usual investment casting methods,* and its flat base was machined approximately perpendicular to the tooth axis.

Particular attention was given to the uniformity of the amalgam and the methods for preparing it, inasmuch as Swartz and Phillips[1] have shown that the strength varies with the amount of mercury expressed, for example. A modified Crandall balance,

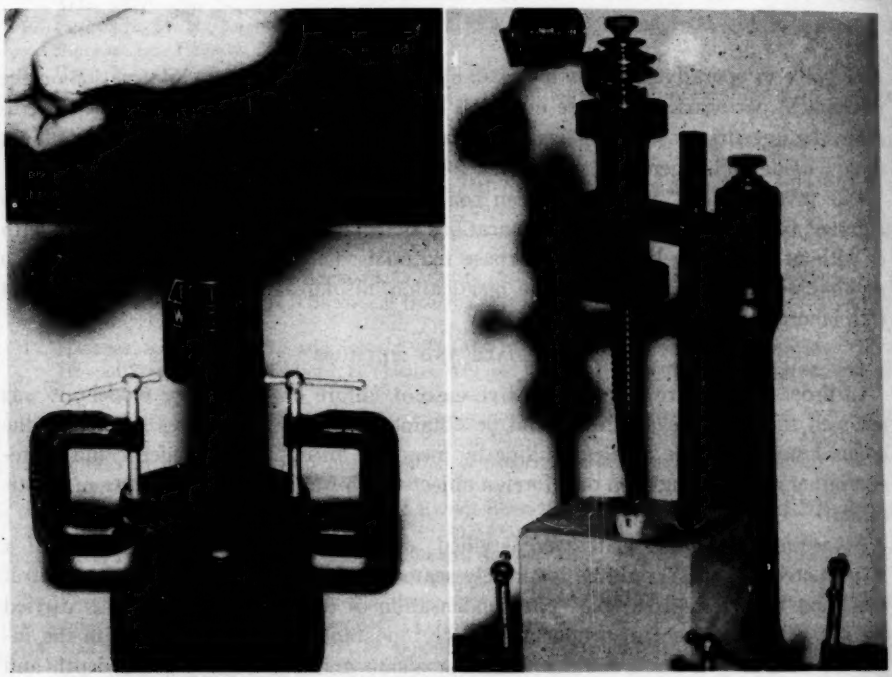

FIG. 1.—Hand press for forming anatomy on amalgam surface. The die is held in place by clamps, and the jig and tooth model are shown below the die (not shown).

FIG. 2.—Prosthetic surveyor modified to take dental handpiece in connection with cavity preparations. The large block of plaster is clamped to the base of the apparatus so that the position of the ivorine tooth can be fixed, and the vertical steel rod serves to stop the rotational movement of the arm in connection with producing the hemispherical depressions. In other steps, the cavity preparations involved hand workmanship, but the depth and vertical marginal walls were made reproducible by presetting the apparatus.

utilizing an extended pointer and a vertical scale, was used for weighing the components. Trituration was for 12 seconds in an amalgamator† for the five parts silver alloy to six parts mercury mixture that was used throughout. The amalgam was not handled with the fingers, in order to avoid contamination by moisture.[2] Condensation by hand appears to be an appropriate method, according to Wilson, Phillips, and Norman,[3] and was used exclusively.

* Tech-Cast, J. F. Jelenko & Co., 136 West Fifty-second St., New York 19, N.Y.

† Wig-l-Bug, Crescent Dental Manufacturing Co., Chicago, Ill.

Ivorine teeth,* model J, were used for all the experiments. The occlusal preparations of these teeth were altered in order to obtain vertical marginal walls and uniform depths by adapting a prosthetic surveyor to hold a dental handpiece and by fixing to this apparatus a jig to hold the teeth in a predetermined position (Fig. 2). Care was used to make certain that the cavity preparations were as uniform as possible by checking the snugness of fit of a metal inlay (Fig. 4). In general, a few thousandths of an inch were considered tolerable.

Two different types of cavity preparations were employed for the tests conducted to determine the point of failure of the fillings with underbase materials. The first had a shallow floor and a hemispherical excavation for the underbase (Fig. 3, *A*); this preparation corresponded with one for which the conical carious lesion extends below the desirable depth of an ideal cavity preparation. The second type (Fig. 3, *B*) had a deep floor essentially perpendicular to the axis of the tooth; this type represented

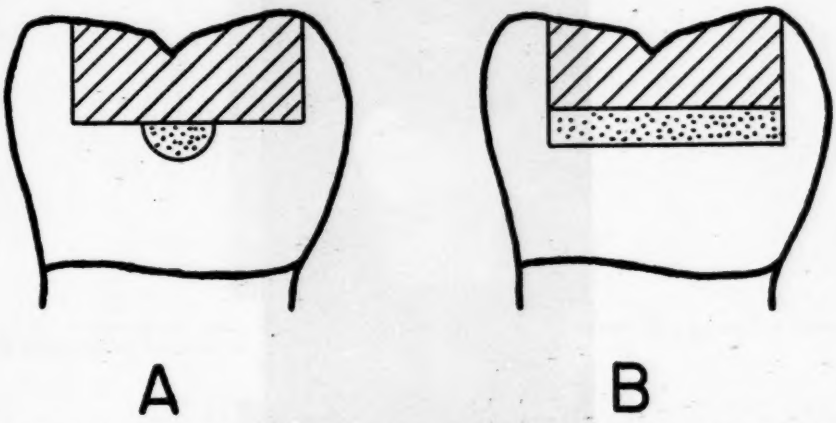

FIG. 3.—Cavity preparations, types *A* and *B*. Cross-sectional views of shallow-cavity (*A*) and deep-cavity (*B*) preparations. Diagonal shading represents amalgam, and stippling represents underbase material. (*Note:* For type *A* restorations with amalgam alone, the hemispherical excavation was absent.)

what would be necessary in case of rampant caries which extends laterally. Similar tests were made on cavity preparations filled solely with amalgam, in which cases the hemispherical depression was not cut for the type *A* cavity preparations. The small excavation was made with a No. 8 round bur and was located approximately below the point where pressure was applied to the restoration.

The loads under which these models failed were measured by a Dillon testing machine, which utilizes mechanical (rather than hydraulic) methods of load application. All loads were applied at a rate of about 50 lbs/sec and were read to the nearest 5-pound mark on the dial. In all tests, except those involving the deep-cavity preparations and the zinc oxide/eugenol underbase, the error in reading the load was less than 1 per cent.

The cavity preparations with a deep floor were filled with base material to the uniform height of 1 mm. above the floor. The amalgam layer, which was condensed upon

* Columbia Dentoform Corp., 131 East Twenty-third St., New York 10, N.Y.

this base, had a minimum thickness of 2 mm. and increased in thickness from this minimum in terms of the anatomy. In order to enhance the set of the zinc oxide/eugenol, a drop of water was placed in each cavity after emplacement of the underbase. It was discovered at the time of testing (48 hours later) that the zinc oxide/eugenol composition had not hardened to a completely rigid condition. (Further comment on this topic will appear below.)

The control models in both cases contained merely amalgam, the only difference being the depth to which excavation was produced. For the deep-floor models (type *B*) the minimum thickness of amalgam was 3 mm., whereas for the shallow-floor models (type *A*) it was 2.3 mm. (No hemispherical depression was excavated for these control specimens.)

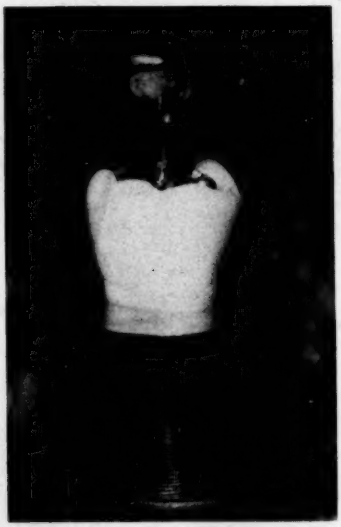

FIG. 4.—Templet or inlay used to check the cavity preparations for uniformity of dimensions

RESULTS AND DISCUSSION

The variation in load at which failure occurred for each type of preparation was found to depend principally upon the reproducibility of the manner of applying the load—i.e., the ability of the operator to seat the steel ball with precision at the intersection of the two channels on the occlusal surface of the model. As mentioned above, the die used to produce the anatomy during the compaction of the amalgam apparently sustained wear, so that later tests did not yield the same reproducibility as that of earlier tests. However, this effect was not of major importance, because earlier tests included all types of preparations, and later tests merely increased the total number of test results, with the condition of the die being essentially comparable for each additional group of preparations.

All tests are summarized in Table 1. No significant differences are noted between the type *A* amalgam filling alone and amalagm filling emplaced above zinc phosphate. The use of zinc oxide/eugenol as an underbase seems to cause a slight reduction in

strength, but the precision of the method of testing probably cannot be trusted with respect to such small differences as 460 versus 500 pounds.

Restorations involving deeper-cavity preparations (type *B*) gave more significant results. Underbases prepared with zinc phosphate sustained far greater loads than those prepared from zinc oxide/eugenol. Examination of the models after failure indicated that the zinc oxide/eugenol preparations had not behaved like rigid materials but had resolved the forces of loading against the marginal walls in such a way that failure of the model was related to the strength of the ivorine tooth. Because of the thinness of the tooth wall, the forces required to produce rupture were consequently far smaller than for the zinc phosphate, which behaved essentially like a non-plastic material.

FIG. 5.—Ivorine tooth with amalgam restoration. The anatomy is shown. The jig used to hold the teeth during testing is shown also, as in Fig. 4.

FIG. 6.—Tooth model after failure. The minimum thickness of the amalgam is twice that of the zinc phosphate cement base.

CONCLUSIONS

Although the conditions of testing of laboratory models cannot be expected to yield data on the absolute strengths of clinical restorations in human teeth, the reduction of the number of independent variables that can be accomplished through the use of such models permits the assemblage of test data of comparative significance.

It has been found that a deep Class I restoration does not have appreciable strength when amalgam is condensed upon a layer of zinc oxide/eugenol, and this type of restoration might fail in clinical practice.

On the other hand, when the zinc oxide/eugenol is used in a small excavation immediately above the pulpal chamber and the principal mass of the amalgam is supported by the tooth structure, the strength can approach that of a base of zinc phosphate or of amalgam alone.

TABLE 1

COMPARATIVE STRENGTHS OF LABORATORY MODELS CONTAINING CLASS I AMALGAM RESTORATIONS WITH AND WITHOUT UNDERBASES

Description of Specimens	All Tests			Acceptable Tests*		
	No.	Average (Pounds)	Limits (Pounds)	No.	Average (Pounds)	Limits (Pounds)
Type A:						
Amalgam only	12	495	360–600	10	500	455–535
Zinc phosphate underbase	24	485	325–645	17	480	390–570
Zinc oxide/eugenol underbase	14	445	335–515	13	460	395–515
Type B:						
Amalgam only	10	355	230–470	8	355	290–420
Zinc phosphate underbase	11	390	335–475	10	380	335–425
Zinc oxide/eugenol underbase	10	120	60–150	9	125	90–150

* Acceptable tests were those within 20 per cent of the average of all tests or 30 pounds (whichever was greater). Averages are recorded to the nearest 5 pounds. Base materials were aged for 24 hours before condensing the amalgam. Tests were made after the amalgam fillings were 24 hours old.

A shallow-cavity preparation utilizing a small depression filled with zinc phosphate appears to have the same strength as does the same cavity preparation (without the depression) when filled solely with amalgam.

For large Class I restorations there appears to be a decrease in strength which accompanies the increase in depth of the cavity preparation even for amalgam alone, but this reduction in strength becomes extreme with the use of zinc oxide/eugenol.

Despite contact with moisture, zinc oxide/eugenol underbases do not set to a rigid condition during 48 hours, and the retained plasticity permits a complex resolution of forces applicable to the marginal walls, so that failure occurs under comparatively small loads.

All of these conclusions have necessarily disregarded the microstructural characteristics of teeth, inasmuch as the tests were applied to homogeneous materials, and are subject to clinical interpretation only to the extent that certain practices probably should be avoided.

SUMMARY

Laboratory models containing both deep- and shallow-cavity preparations were restored (1) with amalgam alone, (2) with underbases of zinc oxide/eugenol, and (3) with underbases of zinc phosphate cement. For the shallow cavity the underbase was emplaced in a small hemispherical excavation, but for the deep cavity the base formed a continuous layer under the amalgam.

Amalgam restorations with underbases of zinc oxide/eugenol failed under relatively small loads if the underbase covered the entire floor of a deep-cavity preparation. When this same base material was confined to a small hemispherical excavation, however, the load sustained was similar to that sustained by zinc phosphate or of amalgam alone.

With increased depth of the cavity preparation, a general decline in strength of the models was observed, even with amalgam alone, and this decline was extreme with zinc oxide/eugenol as a base material.

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Study of Bacteriophage Types and Antibiotic Resistance of Staphylococci Isolated from Dental Students and Faculty Members

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Staphylococcal infections have been included among the most prominent of bacterial diseases for a long time, and in recent years the emergence of antibiotic-resistant strains has created a serious problem in hospitals.[1]

The laboratory study of staphylococci—an interesting and important field for many years—has become even more challenging in recent years partly because of the following points: (*a*) The almost complete correlation of the ability of staphylococci to clot blood plasma with their capacity to produce disease and the corresponding absence of this property in non-pathogenic strains have led to the assumption that coagulase production is closely related to their potential for the establishment of infection.[1] (*b*) Research in bacteriophage typing has proved to be a valuable tool in the study of *Staphylococcus aureus* strains responsible for hospital-acquired infections.[2]

Although several publications[3–5] relative to the importance of staphylococci in the field of dentistry are available, little has been published on bacteriophage types of coagulase-positive strains of staphylococci found in the oral and nasal areas of dental students and dentists. While dental students do not usually have a close association with hospital inpatients, still in a large medical center there is some degree of association between these students and various members of the hospital personnel. In addition, dental students often have some small degree of contact with patients in the dental outpatient department of hospitals. Furthermore, it seems logical to assume that, of the numerous patients visiting a dental clinic, many probably have had some recent experience as hospital patients. Thus the following questions are of interest: (*a*) How does the carrier rate of coagulase-positive staphylococci in general (and the penicillin-resistant strains in particular) compare with similar studies on other groups of individuals? (*b*) Is there an increase in the carrier rate of coagulase-positive staphylococci among dental students after they start clinical work? (*c*) Is there a tendency for any specific phage types of these organisms to predominate in the nasal areas of students and faculty members working under the same clinical conditions?

EXPERIMENTAL METHODS

Nasal swabs obtained from 62 faculty members, 67 Senior students, 70 Juniors, 76 Sophomores, and 80 Freshmen were cultured and studied for staphylococci. These

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samples were obtained by swabbing deeply into the vestibular portion of both nostrils with swabs previously moistened with sterile isotonic sodium chloride solution. The swabs were smeared immediately over the surface of a high-salt-content medium.* The inoculated medium was incubated for 48–72 hours at 37° C., and a search was made for pigmented colonies typical of *S. aureus*. If the latter were found, a colony was transferred to a tube of brain-heart infusion broth† and incubated at 37° C. for 24 hours. If no pigmented colonies were present on the agar plate cultures, a white colony of a staphylococcus was cultured in broth, as noted for the pigmented strains. In order to test for purity, a loopful of each broth culture was streaked on blood agar plates and incubated for 24 hours at 37° C. Pure colonies of staphylococci were then transferred to slants of staphylococcus medium, incubated at 37° C. for 24 hours and kept at 4° C. for future study.

Organisms from overnight nutrient agar growths were inoculated into 1-ml. amounts of fresh citrated rabbit plasma (diluted 1 to 3 with sterile distilled water) and incubated for 6–18 hours in a 37° C. water bath. The coagulase-positive strains were further studied for fermentation of mannitol, sensitivity to antibiotics, and bacteriophage types.

Antibiotic-sensitivity tests were made by streaking 0.1 ml. of a 24-hour brain-veal infusion broth* culture over a nutrient agar plate* with a sterile wire spreader. The plate was allowed to dry from 15 to 20 minutes, and three antibiotic disks‡ were placed on the surface; the plate was incubated at 37° C. for 18–20 hours. The disks included either penicillin (2 units), erythromycin (2 μg.), or tetracycline (5 μg.). If growth occurred in contact with a disk, the organism was recorded as resistant to the antibiotic.

Twenty-nine lyophilized phages§ (3A, 3B, 3C, 6, 7, 29, 39, 42B, 42D, 42E, 44A, 47, 47C, 52, 52A, 53, 54, 55, 70, 71, 73, 75, 77, 79, 80, 81, VA_4, 187, and 523) were used for phage typings. The specificity of all the phages except 71 and 187 was tested by checking them against known bacteriophage-type strains.||

Agar plates containing 20 ml. of typticase soy agar (BBL) were poured and left overnight at 37° C. The bottom of each plate was divided into sections and numbered with a code for a phage type. The agar was streaked with an 18-hour typticase soy broth (BBL) culture using sterile swabs; this was allowed to dry for 20 minutes, and then phages were placed over their corresponding numbers by use of 1-ml. tuberculin syringes and 27-gauge 1-inch needles. The plates were incubated for 18–24 hours at 30° C. and examined by transmitted light. Areas which showed either confluent lysis or over 50 plaques were considered as positive reactions.

RESULTS

Non-pigmented colonies of staphylococci were present in all cultures made from nasal swabs obtained from 355 students and faculty members of the Medical College of Virginia, School of Dentistry. Pigmented colonies of staphylococci were obtained

* Staphylococcus Medium #110, Difco Laboratories, Inc.

† Difco Laboratories, Inc.

‡ Difco Laboratories, Inc.

§ Obtained from Microbiological Associates, Inc., Washington, D.C.

|| Obtained from the Microbiology Laboratory of the Medical College of Virginia Hospital.

from 163 of the nasal swabs; of these, 143 coagulated rabbit plasma. In contrast, of 192 strains from non-pigmented colonies, only 7 were coagulase-positive.

Only 10 (5 pigmented and 5 non-pigmented) of the 150 coagulase-positive strains failed to ferment mannitol. Since 2 from each group of non-mannitol fermenters were sensitive to staphylococcal bacteriophages, all were included in strains listed in this study.

Of the 150 strains, 62 (41.3 per cent) were resistant to penicillin, while only 8 (5.3 per cent) were resistant to tetracycline and 2 (1.3 per cent) were resistant to erythromycin.

As noted in Table 1, coagulase-positive strains of staphylococci were isolated from 42.3 per cent of the 355 subjects. In contrast, penicillin-resistant, coagulase-positive strains were isolated from only 17.5 per cent of the same subjects.

TABLE 1

INCIDENCE AND PENICILLIN RESISTANCE OF COAGULASE-POSITIVE STAPHYLOCOCCI IN NASAL SWABS FROM 355 DENTAL FACULTY MEMBERS AND STUDENTS

Subjects	No. in Group	Coagulase-positive Strains			
		Number	Per Cent of Number in Group	Strains Resistant to Penicillin	
				Number	Per Cent of Number in Group
Freshmen	80	29	36.3	12	15
Sophomores	76	32	42.1	10	13.2
Juniors	70	33	47.1	9	12.9
Seniors	67	30	44.8	15	22.5
Faculty	62	26	41.9	16	25.8
Total	355	150	42.3	62	17.5

A statistical analysis of data included in Table 1 was made by means of a standard chi-square test of contingency, in order to determine whether the carrier rate of staphylococci listed was significantly different among the five groups of subjects.[6] This test was used for both the coagulase-positive strains in general and the penicillin-resistant strains in particular. According to the results, there were no differences significant at the 0.05 level among the five groups in either case.

Since the ratios for penicillin-resistant strains seemed to fall into two divisions, a two-by-two chi-square test[6] was applied, to test the possibility that Freshmen, Sophomores, and Juniors, as compared with Seniors and faculty members, would demonstrate a difference. Again there was no significant difference at the 0.05 level.

Table 2 illustrates a relatively wide scattering of phage types within each broad group. The chief exception is in cases of strains sensitive to VA_4 phage, as noted with faculty members and Seniors. For examples, strains sensitive to VA_4 alone or with other phages accounted for 8 of 9 Group 3 strains for faculty members and 5 of 7 Seniors. Strains sensitive to 80/81 phage combinations alone or with other phages

TABLE 2

GROUPING OF COAGULASE-POSITIVE STAPHYLOCOCCI ACCORDING TO PHAGE TYPES

PHAGE GROUPS	GROUPS OF PARTICIPANTS	TOTAL
	A. Freshmen (29 Strains)	
I	79, 80, 52/80, 52A/79 (2)*	5
II	3A (2), 3C, 39/55/71	4
III	VA$_4$, 6/7, 6/47/53/75, 47/53/54/75/VA$_4$, 75	5
IV	NONE	.....
Misc.	52/52A/80/81 (2)	2
Non-typable	.....	13
	B. Sophomores (32 Strains)	
I	52A, 52/80 (4), 52/52A/80, 79	7
II	3A, 3C, 55	3
III	VA$_4$ (3), 6, 7, 70, 42B, 7/47/70	8
IV	42D	1
Misc.	81, 7/81, 80/81 (3), 6/42E/52	.....
	44A/52, 44A/52/52A, 7/42D/42E/52/54/70/75	9
Non-typable	.....	4
	C. Juniors (33 Strains)	
I	29, 80, 52/80 (2)	4
II	3A, 71, 55/523, 3A/39/55, 3A/55/71	5
III	7, 6/53/54/VA$_4$	2
IV	NONE	.....
Misc.	80/81, 52/80/VA$_4$, 187	3
Non-typable	.....	19
	D. Seniors (30 Strains)	
I	52 (2), 52A, 80	4
II	3A (2)	2
III	VA$_4$ (3), 7, 77/VA$_4$, 6/53/VA$_4$, 47/53/54/75	7
IV	NONE	.....
Misc.	47C, 80/81, 6/42D/47/47C/52/54	3
Non-typable	.....	14
	E. Faculty (26 Strains)	
I	29, 52A/79, 52/80, 52/52A/80, 29/52/52A	5
II	3A (2), 523	3
III	VA$_4$ (5), 7, 7/VA$_4$, 7/77/VA$_4$, 6/7/42E/77/VA$_4$	9
IV	NONE	.....
Misc.	52/80/81 (2)	2
Non-typable	.....	7

* Figures within parentheses indicate total number of strains.

occurred nine times and were relatively well distributed between the five groups of subjects. Strains sensitive to 80 or 81 alone or in various combinations with other phages accounted for 25 strains, 10 from the Sophomores and the remaining well distributed between the other four groups of subjects.

DISCUSSION

The finding of coagulase-positive staphylococci in cultures from nasal swabs of 42.3 per cent of 355 dental students and faculty members is not surprising, since, as mentioned in an extensive review by Gosling and Büchli,[7] investigators have shown that about 30–60 per cent of normal persons carry potentially pathogenic staphylococci in their vestibulum nasi without evidence of a clinical infection.

The latter review also includes reports of from 2 to 16 per cent incidence of penicillin-resistant, coagulase-positive stains of staphylococci in nasal swab cultures from the population at large. In a more recent review,[8] reports include as high as a 25 per cent incidence of these organisms in nasal areas of non-hospitalized individuals. Thus the 12.9–25.8 per cent of similar organisms reported in Table 1 may be considered within the range of figures given for other non-hospitalized groups.

The 36.3 per cent of Freshmen harboring coagulase-positive *S. aureus* stains was only 8.5 per cent less than that found for Seniors. Based on a statistical analysis, this was not a significant increase. Furthermore, the 42.1 per cent incidence for Sophomores with no contact with clinical practice was very similar to the percentages reported for dental students and faculty members engaged in clinical practice.

There was some increase in the 22.5–25.8 per cent incidence of penicillin-resistant strains for Seniors and faculty members as compared with the 12.9–15 per cent for Freshmen, Sophomores, and Juniors. When subjected to a statistical analysis, the difference noted was not significant.

The results listed in Table 2 show a wide scattering of phage types within each broad grouping. This is similar in general to results found by Taplin and Goldsworthy[9] in a study of 225 strains of staphylococci isolated from the mouths of 130 healthy children. The only suggestion of a predominance of a given phage type was with the VA_4 strains, in Group 3, for Seniors and faculty members. Strains sensitive to VA_4 only were found for 3 of 67 Seniors and 5 of 62 faculty members. If, however, strains sensitive to VA_4 with and without sensitivity to other phages are considered, an incidence of 5/67 (7.4 per cent) is noted for Seniors and 8/62 (13 per cent) for faculty members. Since there is no reason for assuming that strains sensitive to different combinations of phages are similar, no conclusion can be drawn from these data.

The 80/81 strains so often found in epidemic infections in hospitals[2] were isolated from 9 individuals and were relatively evenly distributed among the five groups.

These data do not seem to suggest that dental students or dentists in this location harbor potentially pathogenic staphylococci to any greater extent than has been reported for other groups without definite hospital connections. It does, however, agree with other investigations[3–5] that antibiotic-resistant strains of *S. aureus* must be considered potential sources of contamination in dental operations.

SUMMARY

Coagulase-positive staphylococci were isolated from nasal areas of 42.3 per cent of 355 dental students and faculty members. The incidence of penicillin-resistant, coagulase-positive staphylococci for the same group was 17.5 per cent.

There was no evidence of a statistically significant increase in coagulase-positive staphylococci in general or penicillin-resistant strains in particular, in comparing Freshmen and Sophomores with Juniors, Seniors, and faculty members.

The author wishes to acknowledge the assistance of Dr. M. E. Turner, of the Department of Biophysics and Biometry, who prepared the statistical analysis.

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*Localized Cytomegalic Inclusion Disease of Lacrimal Glands in the NMRI-D Strain Caries-susceptible Rat**

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In a previous report,[1] the presence of a localized cytomegalic inclusion disease in exorbital and intraorbital lacrimal glands of male laboratory rats was described. This condition was observed in adult males of eight laboratory colonies but was not seen in any wild urban or rural Norway rats. A brief summary of this report is in order.

For a recent toxicological study (unpublished), several hunded adult male Sprague-Dawley rats were purchased from a midwestern firm. Routine histologic survey of tissues from the head and neck showed invasion of lacrimal glands by a cytomegalic inclusion disease. All other general body-organ systems were free of inclusion involvement. Certain distinctive features described by Wyatt[2] as essential to complete morphologic diagnosis were satisfied, namely, eosinophilic inclusion bodies, margination of nuclear chromatin, and specific cellular gigantism. For comparative purposes, attempts were made to procure normal lacrimal tissue. Six adult male and female rats of various ages from each strain of Naval Medical Research Institute stock, i.e., Long-Evans, Sprague-Dawley, and the closed colony NMRI-Dental Caries-Susceptible, were selected at random and prepared for histologic evaluation. All strains showed extensive lacrimal involvement by inclusion disease but only in males. Again, all other organ systems were free of any cytomegalic inclusion disease. Sample male rats, four to each group, were procured from four other laboratories in various geogaphic locations in the United States. Inclusion disease was present in all lacrimal glands, although variations in inclusion morphology and lesion severity were observed.

A group of six wild urban male and six rural Norway rats, trapped in the Baltimore area, were free of lacrimal involvement. However, urban rats possessed intranuclear inclusions within kidney tubular cells, similar to the condition previously reported by Hindle.[3] Salivary gland virus disease (SGV), as described by Kuttner[4] and Smith,[5] was present in acinar epithelium of submandibular salivary glands and in ductular cells of sublingual glands of farm rats.

An extensive survey of the literature indicated that the presence of cytomegalic inclusion disease in lacrimal glands of the male laboratory rat was a new observation. Second, the following references did not reveal any type of inclusion disease that produced similar lesions. The occurrence of cytomegalic inclusion disease and/or salivary

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* The opinions or assertions contained herein are the private ones of the writer and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

gland virus disease has been reported previously in humans,[2, 4, 5–15] monkeys,[16–19] dogs,[20–21] marsupials,[22] rabbits,[23] and rodents.[24–26] Since lacrimal gland inclusion disease had not been studied previously, a short-term investigation was undertaken to observe the onset, progressive tissue changes, and ultimate effect of localized lacrimal gland inclusion disease in the closed colony NMRI-D strain rat. Furthermore, since it appears that this condition is similar to salivary gland virus disease, it was considered imperative that a survey be made of our caries-susceptible colony for possible detection of SGV disease in the salivary system.

MATERIALS AND METHODS

Sample male and female rats from the closed colony NMRI-D strain, caries-susceptible,[27] were examined at 50-day intervals, starting at birth, to record progressive changes in lacrimal pathology and for survey of SGV disease. Since our earlier observations showed all females to be free of lacrimal disease, two females of identical age were used as comparative controls for each three experimental male rats. All animals were sacrificed by chloroform inhalation. In addition to lacrimal gland dissections, the salivaries, including minor glands from tongue and floor of mouth, lung, kidney, liver, spleen, stomach, intestines, and bladder were taken for histologic examination. All tissues were fixed in Bouin's solution, processed in paraffin, and stained with hematoxylin and eosin.

Since Wyatt[2] in his description of human cytomegalic inclusion disease mentions "that the virus may pass the placental barrier is indicated by the number of still-born infants in which it is found," uteri and placentae were procured from three mothers at full term before commencement of labor.

RESULTS

The presence of cytomegalic inclusion disease was limited entirely to exorbital and intraorbital lacrimal glands of the male rat. No other organ systems were involved. All females were free of lacrimal gland infection. No evidence of inclusion disease was seen in uteri and placentae obtained at term. The following observations show the effect of this disease on lacrimal gland morphology.

Birth to 50 days.—No inclusion bodies were present in the newborn. Intraorbital and exorbital lacrimal glands were incompletely formed. Vigorous growth was occurring, and mitotic figures were numerous. At 50 days of age, Figure 1, an occasional eosinophilic inclusion was observed. The distinct halo was not present, and there was little margination of nuclear chromatin. There was no apparent cytomegaly or round-cell infiltrate.

50 to 150 days.—As the rat continued to mature, subsequent growth and enlargement occurred in both pairs of lacrimal glands. During this period, there was an increase in number of intranuclear inclusions. Initial cytomegaly of affected acinar cells was occasionally observed. Margination of nuclear chromatin was evident. Some round-cell infiltration was seen in the supporting stroma.

150 to 250 days.—Many intranuclear inclusions had undergone distinct morphologic changes. Some appeared as enlarged spherical, granular-like masses, completely filling the nucleus (Fig. 2). The nucleus appeared void of chromatin, and the characteristic halo was absent. Some nuclei had two well-formed inclusion bodies. Cyto-

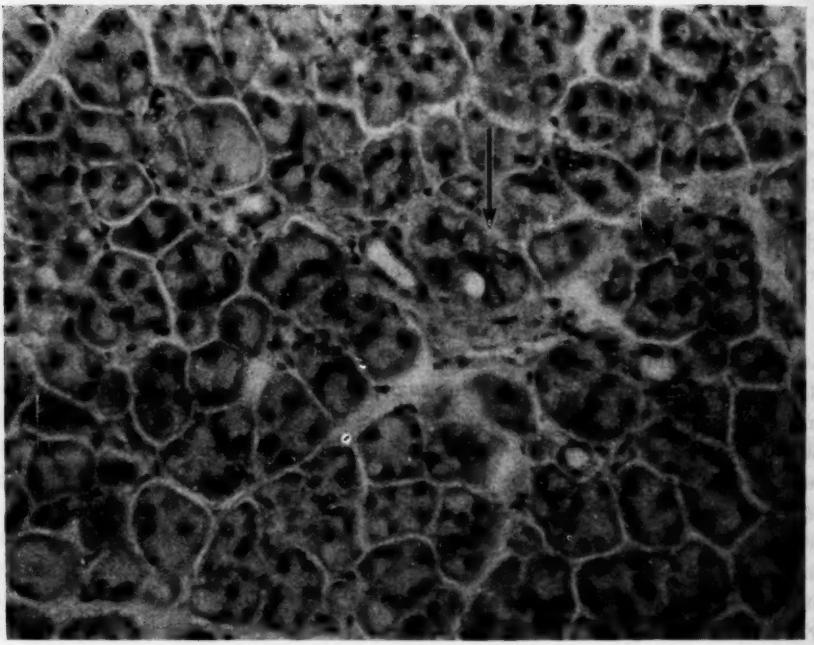

FIG. 1.—Initial visible onset of cytomegalic inclusion disease. Exorbital lacrimal gland from 50-day-old NMRI-D strain male. An occasional intranuclear inclusion body and nuclear enlargement is seen at tip of black arrow. (Hematoxylin and eosin; orig. mag. ×190.)

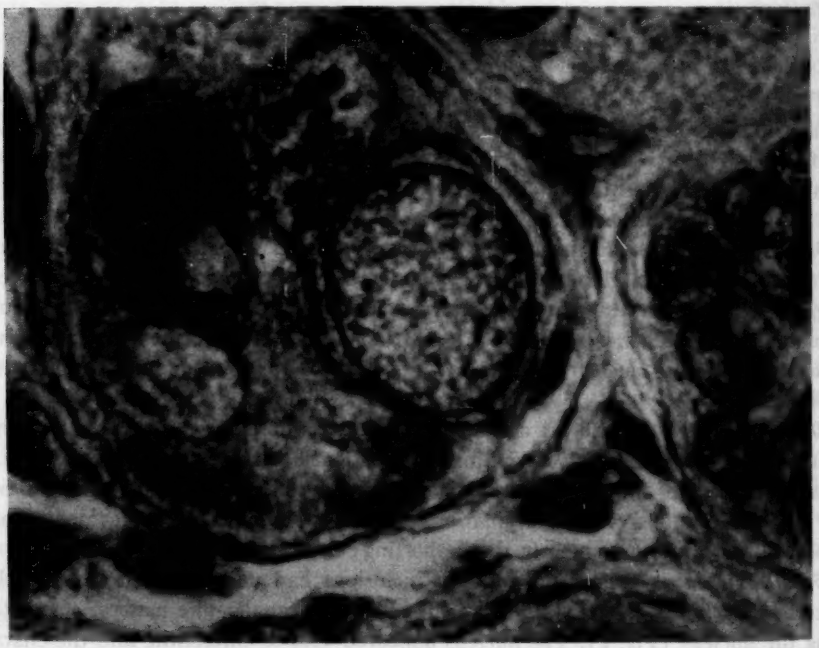

FIG. 2.—Granular eosinophilic intranuclear inclusion body. Exorbital lacrimal gland, 150-day-old NRMI-D strain male; on the left, a smaller inclusion body is visible in the nucleus. Hematoxylin and eosin; orig. mag. ×800.)

megaly of affected acinar cells continued to be more apparent. Nuclear swelling and enlargement was evident, with some nuclei exhibiting resultant pleomorphism.

250 to 350 days.—The peak of the disease process was reached at 300–350 days (Fig. 3). Affected acinar cells exhibited marked pleomorphism and subsequent cytomegaly. Bizarre nuclear patterns were noted, namely, asymmetrical multipolar mitotic figures (Figs. 4 and 5), syncytial massing, and giant hyperchromatic club-shaped nuclei (Fig. 6). Interstitial tissue (Fig. 3) showed a marked increase in stromal cells and lymphocytic infiltration. Both intraorbital and exorbital lacrimal glands exhibited the same degree of involvement. No evidence of inclusion disease was present in female lacrimal tissues (Fig. 7).

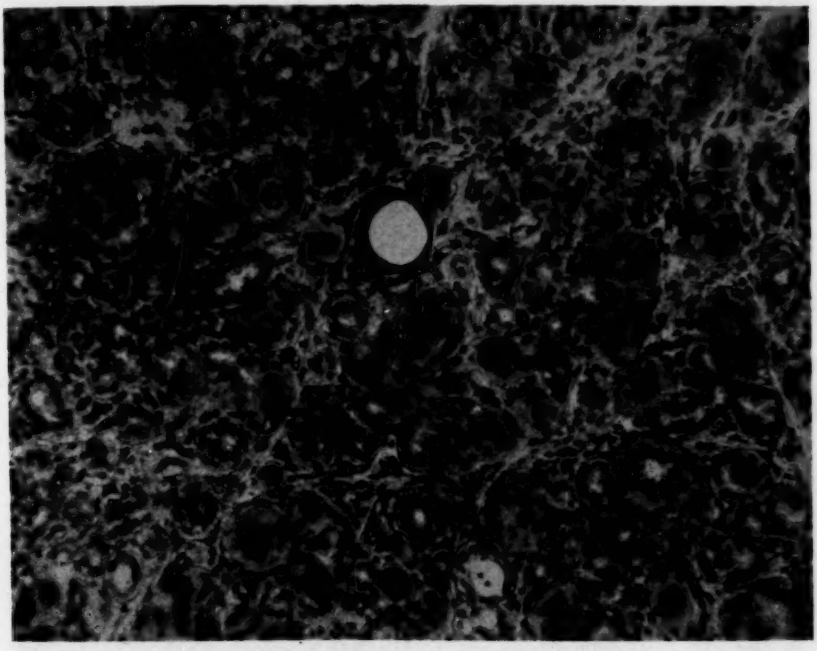

FIG. 3.—Intraorbital lacrimal gland from a 350-day-old male. Note bizarre architecture and lymphocytic infiltration. (Hematoxylin and eosin; orig. mag. ×190.)

350 days to senility.—During this period, lacrimal tissues had undergone gradual degeneration and necrosis. In 450- and 500-day-old males, many areas of lacrimal tissue in both exorbital and intraorbital glands were replaced by sebaceous acini similar in morphology to the Harderian gland (Fig. 8).

Variations in inclusion morphology.—Four littermate pairs, males and females, 200–250 days old, of the Sprague-Dawley strain were obtained from another laboratory for lacrimal gland examination. Several interesting variations in inclusion morphology were observed (Fig. 9). The inclusion body at the top of the picture completely fills the nucleus, while the adjacent nucleus, though void of an inclusion body, appears to be degenerating. A large atypical inclusion body, 23 μ in diameter, is present at the bottom of Figure 9. Several adjacent nuclei are undergoing compression (Fig. 10).

FIG. 4.—Cytomegalic acinar cell with tripolar mitotic figure. Note pleomorphic cell outline. Intraorbital lacrimal gland, 350-day-old NMRI-D male. (Hematoxylin and eosin; orig. mag. ×900.)

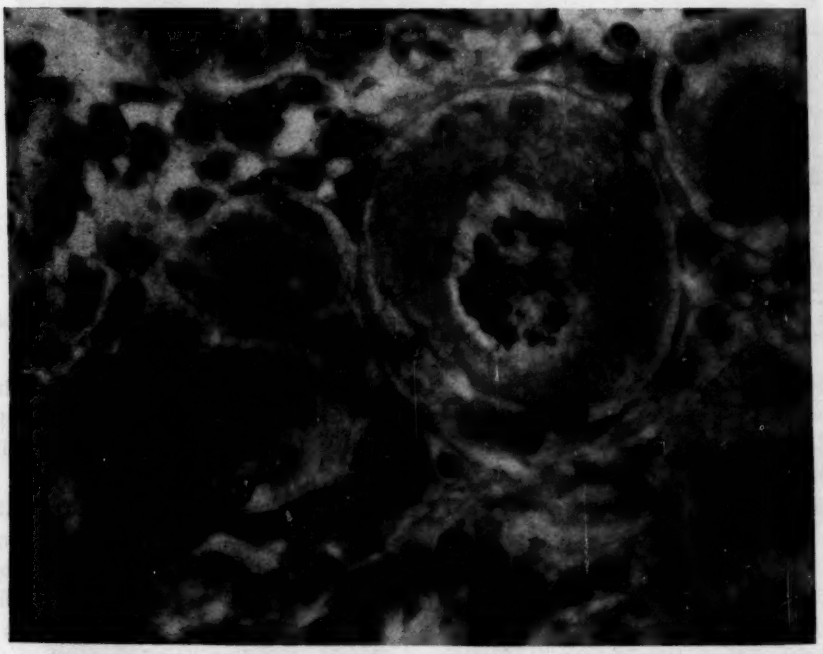

FIG. 5.—Cytomegalic acinar cell showing bizarre mitotic activity. Unaffected acinar cells of normal size can be seen at the bottom center of the photograph. Intraorbital lacrimal gland, 350-day-old NMRI-D strain male. (Hematoxylin and eosin; orig. mag. ×800.)

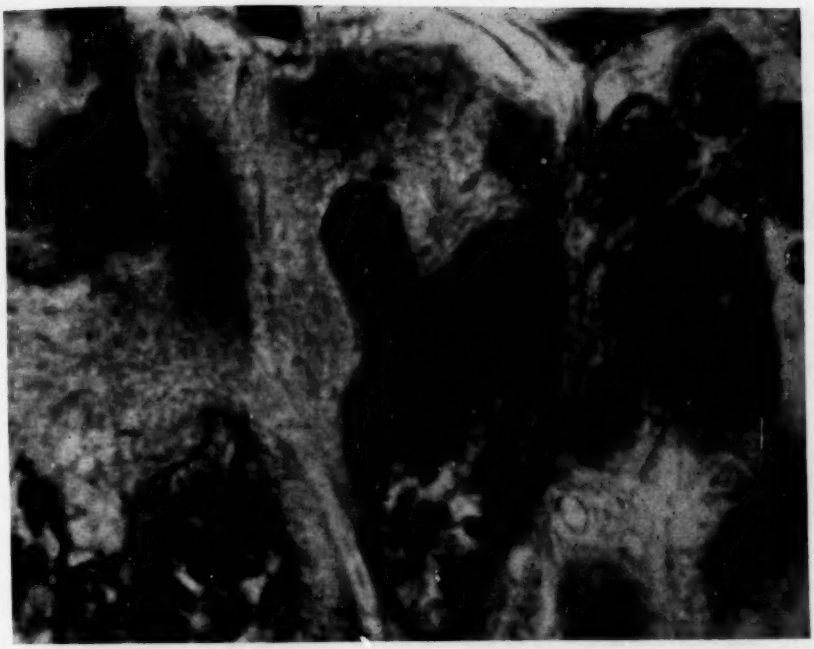

FIG. 6.—Cytomegalic acinar cell with hyperchromatic club-shaped nucleus. The nucleus appears to be undergoing fission. Note the hypochromatic nucleus immediately below. Intraorbital lacrimal gland, 350-day-old NMRI-D strain male. (Hematoxylin and eosin; orig. mag. ×800.)

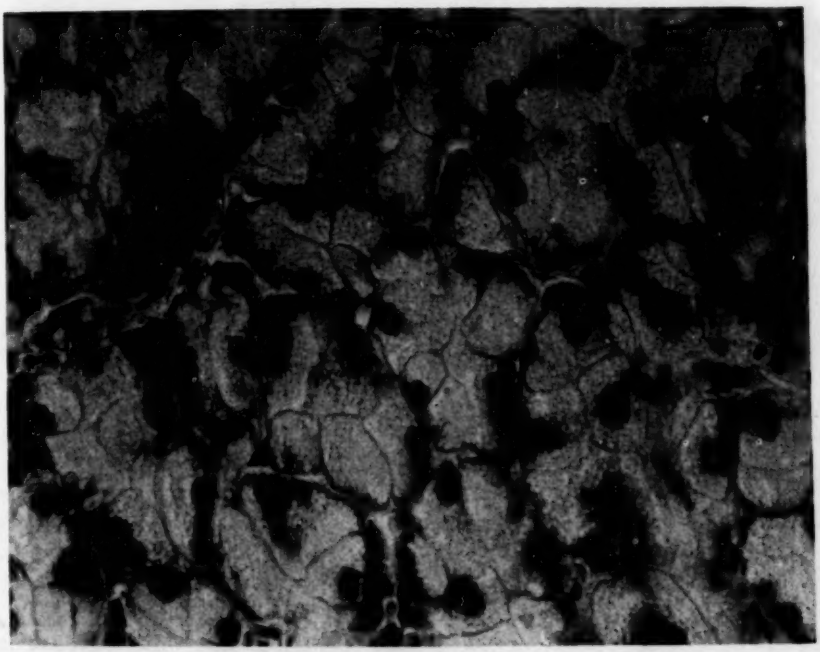

FIG. 7.—Inclusion disease-free exorbital lacrimal acini from NMRI-D strain female, 300 days of age. (Hematoxylin and eosin; orig. mag. ×250.)

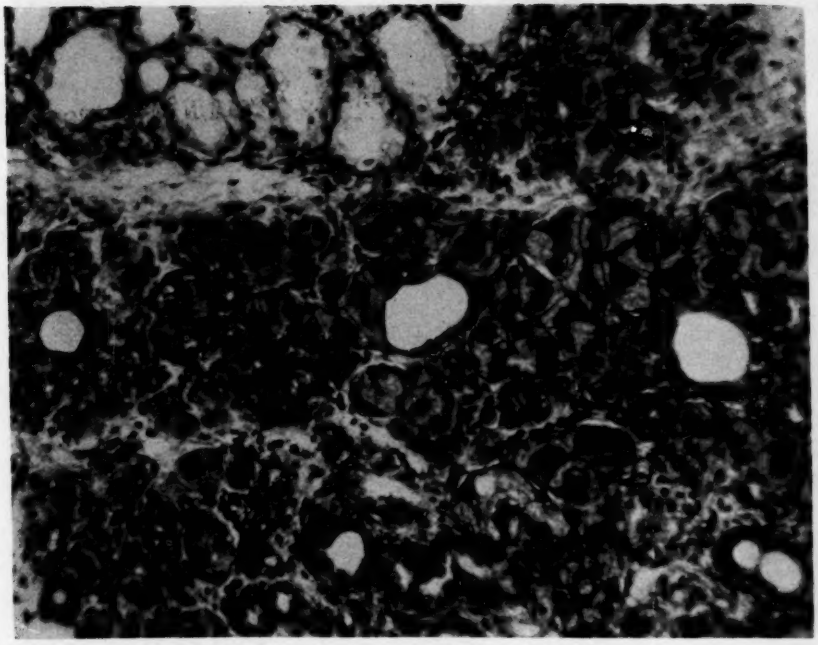

FIG. 8.—Advanced cytomegalic inclusion disease, exorbital gland in a 500-day-old NMRI-D strain male. Note the presence of Harderian-like acini in the upper portion of the photograph. (Hematoxylin and eosin; orig. mag. ×190.)

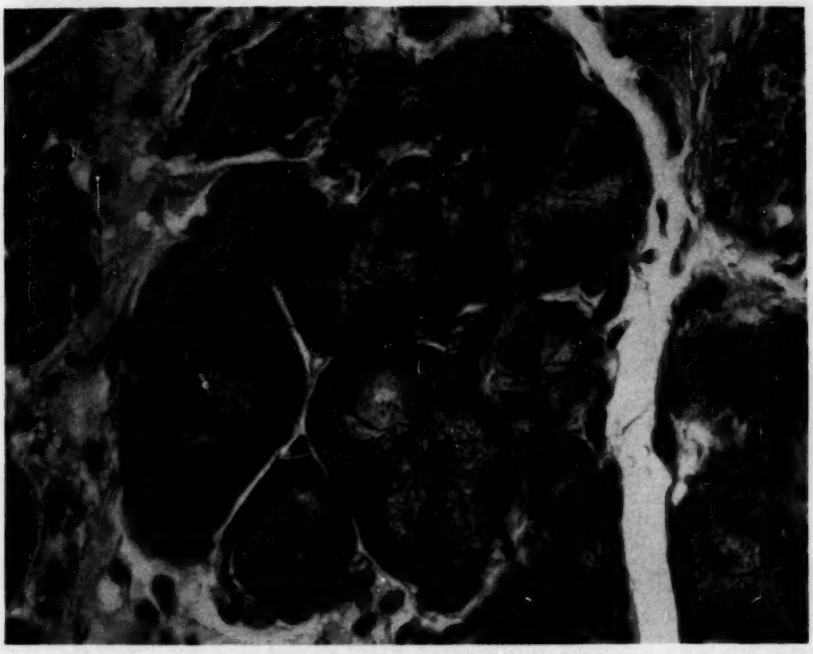

FIG. 9.—Cytomegalic inclusion disease in exorbital lacrimal gland of a 250-day-old Sprague-Dawley male rat. The large inclusion body at the left center bottom of the photograph was not seen in the NMRI-D strain rat. (Hematoxylin and eosin; orig. mag. ×250.)

Note also, within this structure, several basophilic spherical masses that seem to resemble chromatin granules. None of these types of inclusions were observed in the NMRI-D strain rat. Like all other colonies examined, inclusion disease was not present in females of this strain.

DISCUSSION

A comparison between lesions in this lacrimal gland disease of male laboratory rats and those of salivary gland virus disease reveals several differences. If we compare inclusion morphology as shown in Figure 11 (SGV disease in submaxillary gland of an NMRI woodchuck) with the inclusion body shown in Figure 12 (intraorbital lacrimal

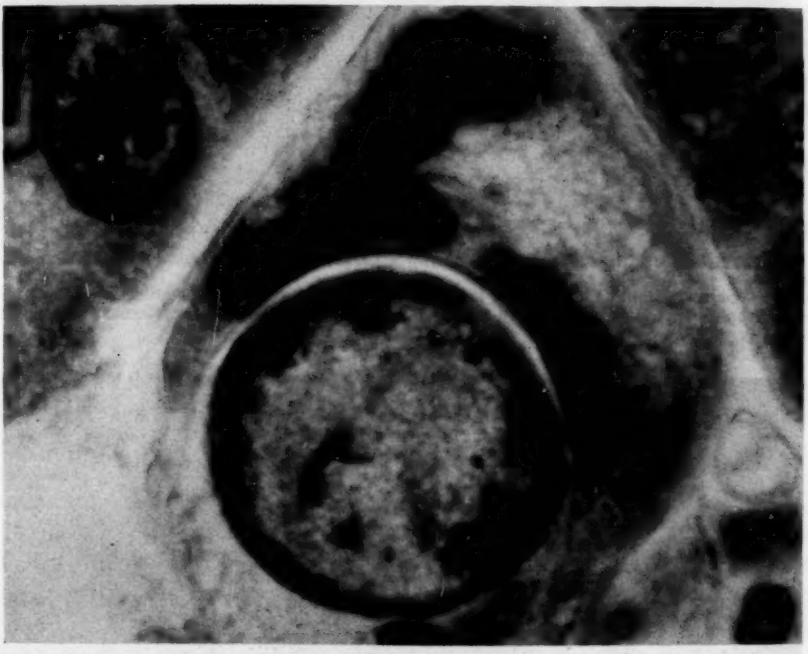

FIG. 10.—Enlarged view of an inclusion body, 23 μ in diameter, of Fig. 9. Note compression of adjacent nuclei. It is difficult to say whether the location is intra- or extracellular. (Hematoxylin and eosin; orig. mag. ×900.)

gland of an adult NMRI-D strain rat), certain differences are apparent. Note that in Figure 11—true SGV disease—intranuclear inclusions appear ovoid. Spherical basophilic cytoplasmic inclusions have been extruded into the lumen of the acinus. In Figure 12 the inclusion body is more spherical, with finer granulations. Margination of nuclear chromatin is prominent, while absent in Figure 11. Basophilic cytoplasmic inclusions were never observed in lacrimal gland disease.

Another factor distinguishing this lacrimal disease from SGV disease was the effect on cell nuclei. In 300–400-day-old males, two distinct nuclear conditions were present. In one aspect, many nuclei showed typical eosinophilic intranuclear inclusions with subsequent cytomegaly of acinar cells. Chromatin appeared to be compressed and

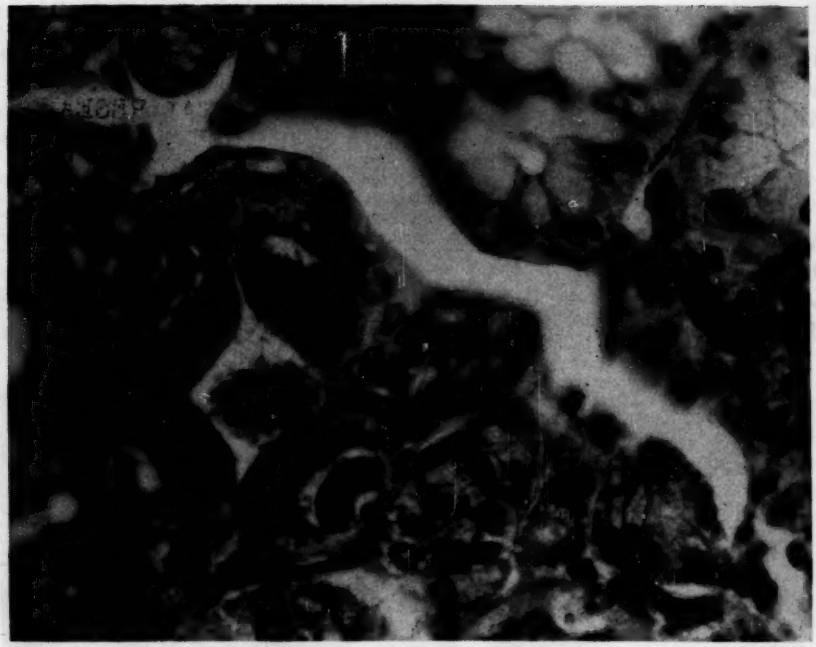

FIG. 11.—Salivary gland virus disease in submaxillary gland of NMRI woodchuck. Note the presence of basophilic cytoplasmic inclusion bodies in the lumen of the alveolus. (Hematoxylin and eosin; orig. mag. ×250.)

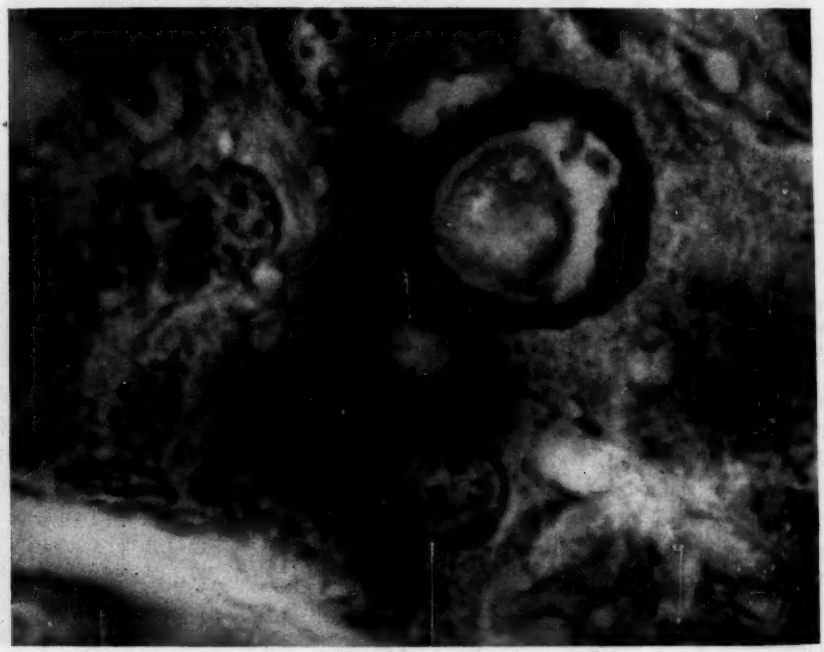

FIG. 12.—Lacrimal gland inclusion disease. The intranuclear inclusion body is finely granular. Note characteristic halo and margination of nuclear chromatin. Basophilic cytoplasmic inclusions are absent. (Hematoxylin and eosin; orig. mag. ×800.)

limited to the periphery of the nuclear membrane. Other nuclei, apparently free of any inclusions, showed vigorous forms of atypical mitotic activity (Figs. 4, 5, and 6). Cytomegaly was present. Note in Figure 5 the irregular positioning and size of the chromosomes. In Figure 4 a tripolar mitotic figure is shown in anaphase. In Figure 6 the nucleus appears to be splitting by direct nuclear division. Mitotic activity was limited entirely to the nucleus, whereas division of the cytoplasm was not observed. Nuclear division was never observed in any acinar or ductular cell possessing an inclusion body.

The only report in the literature showing somewhat similar bizarre inclusion morphology and concurrent mitotic activity was the description by Hurst[22] of intranuclear inclusion disease in the kidneys of Australian opossums kept under hygienic laboratory conditions.

An unusual manifestation observed in degenerating exorbital lacrimal glands of all 450- to 600-day-old males was the presence of sebaceous acini similar to Harderian gland tissue (Fig. 8). These areas were visible during gross dissection of the gland, appearing as white patches within the parenchymal tissues.

Since salivary gland virus disease was not present in any of the salivary tissue, it is doubtful whether this lacrimal gland infestation by an unknown type of localized cytomegalic inclusion disease effects significantly the results of our caries studies with the NMRI-D rat. It is suggested that all investigators survey their colonies for the presence of SGV and lacrimal inclusion disease.

SUMMARY

The morphologic effects of a cytomegalic inclusion disease in exorbital and intraorbital lacrimal glands of the NMRI-D caries-susceptible rats was observed at 50-day intervals starting at birth and continued for 600 days. In 350-day-old animals, affected acinar cells exhibited marked pleomorphism and subsequent cytomegaly. Bizarre nuclear patterns were noted, namely, asymmetrical multipolar mitotic figures, syncytial masses, and giant hyperchromatic club-shaped nuclei containing one to four eosinophilic inclusions. The presence of intranuclear inclusions and subsequent bizarre cellular activity was not observed in any females, regardless of age. The salivary system was free of any inclusion disease.

The authors wish to take this opportunity to express their appreciation to K. F. Glassford, DT1, U.S.N., and R. A. Little, D.N., U.S.N., for their valuable technical assistance.

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Experimental Caries and Gingival Pathologic Changes in the Gnotobiotic Rat

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Dental caries has been one of the most widely studied diseases of mankind, but it was not until 1954 that Orland, Blayney, Harrison, Reyniers, Trexler, Wagner, Gordon, and Luckey[1] using germ-free rats maintained on a cariogenic diet provided unequivocal evidence that experimental caries does not occur in the absence of micro-organisms. In carefully controlled experiments these workers demonstrated that, whereas conventional rats in a normal environment developed extensive caries, germ-free rats maintained on the same cariogenic diet remained caries-free. Shortly afterward Orland, Blayney, Harrison, Reyniers, Trexler, Ervin, Gordon, and Wagner[2] reported the production of dental caries in gnotobiotic* rats infected with combinations of an enterococcus and a proteolytic bacillus or an enterococcus and an anaerobic pleomorphic bacterium.

In the present study we have confirmed and extended the basic observations of Orland *et al.*,[1, 2] using a different cariogenic diet and a single infecting organism.

EXPERIMENTAL METHODS

Sixteen weanling white rats of the Lobund germ-free strain ranging from 25 to 30 days in age were divided into two groups and housed in Reyniers germ-free isolators of the type employed by Orland.[1, 2] Group I consisted of five males and five females, and Group II of two males and four females. The animals were distributed two to a cage, one male and one female, except for one cage in Group II which contained two females. The animals in Group I were inoculated with a streptococcus isolated from the oral cavity of the rat, while those in Group II remained as a germ-free control group. A third group of rats used as a conventional control group consisted of a litter of three male and five female weanlings from a pair of breeders originally of the Lobund germ-free strain but brought to a conventional environment prior to mating.

The diet used was based on cariogenic diet 585 previously described in studies of the effects of antibiotics on experimental caries in rats.[3] Autoclaving diet 585 increased its cariogenic potential for convential rats but severely impaired its capacity to maintain these animals at a normal rate of growth.[4] Fortification of this diet with 10 per

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* The term "gnotobiotic" is used here to describe an otherwise germ-free animal bearing a known microbial flora. Thus, inoculated animals are gnotobiotic. While, strictly speaking, animals carrying no microbal flora are also gnotobiotic, we prefer to use the term "germ-free" in this paper to avoid confusion. The term "conventional" animal refers to animals born and reared in the usual animal quarter environment and presumably carrying a normal microflora.

cent "vitamin-free" casein and with Gustafsson's vitamin supplement[5] prior to autoclaving resulted in growth rates approximately two-thirds that observed in conventional animals maintained on the unautoclaved diet.

The pattern of caries obtained in conventional animals maintained on the autoclaved supplemented diet, referred to hereafter as "diet 585 VC," was very similar to that normally seen with diet 585. This consisted of extensive cavitation of the occlusal sulci, occurring primarily in the first and second mandibular molars but also observed, although with less frequency, in any of the molar sulci. Occasionally smooth-surface lesions of the buccal or lingual molar surfaces were also noted.

When diet 585 VC was autoclaved, it turned dark brown and tended to cake. Before it was dispensed into the food cups, it was ground to approximately its former particle size by passing it through a small hand grater kept in each of the germ-free tanks. The diet for the conventional controls was treated in the same way. Sterilization of the diet was accomplished by autoclaving at 122° C. for 30 minutes in open pans. The depth of the layer of diet was never more than ½ inch. Diet and distilled water were available ad libitum.

The strain of streptococcus used to inoculate the germ-free rats has not been specifically identified. It is a micro-aerophilic α-hemolytic streptococcus which was isolated from swabbings of the teeth of a rat receiving cariogenic diet 585, and it or similar organisms have been routinely observed by us in oral or fecal samples of rats receiving this diet. Surface colonies are gray-white, opaque, and have a raised irregular center usually surrounded by a papilliform ring (Fig. 1, *A*). Subsurface colonies show a characteristic bread-crumb appearance (Fig. 1, *B*). Colony size at 48 hours ranges from 0.5 to 2.0 mm. on adequate media when cultures are incubated at 37° C. under 95 per cent N_2–5 per cent CO_2. In an aerobic atmosphere, growth occurs more slowly than under microaerophilic conditions.

Colonies on Chapman's tellurite medium[6] are dark brown to black in color, raised and rugose, and range from 0.5 to 1.0 mm. in diameter. On this medium, as on 5 per cent sucrose blood agar, the texture of the growth is firm, almost rubbery. The growth on Chapman's medium appears to be intermediate between that of *Streptococcus salivarius* and *S. faecalis* but is easily distinguishable from either type.

The organism is a Gram-positive, non-motile streptococcus which can form long chains of paired round to slightly oval cells in broth media. It does not reduce nitrate or produce indole, catalase, gelatinase, chondroitinase, or collagenase. Growth in the presence of 4 per cent NaCl is weak and is absent in media containing 6.5 per cent NaCl. Growth occurs in media containing 10 per cent bile, but not 40 per cent bile. Litmus milk and 0.1 per cent methylene blue milk are curdled and reduced but not peptonized. The organism produces acid but not gas in 24–48 hours from glucose, sucrose, lactose, levulose, galactose, maltose, mannose, mannitol, melibiose, cellobiose, raffinose, sorbitol, trehalose, inulin, and salicin. Adonitol, arabinose, dulcitol, glycerol, inositol, melezitose, rhamnose, sorbose and xylose are not fermented within 2 weeks. Esculin was split but α-methylglucoside, α-methylmannoside, hippurate, and starch were not hydrolyzed. The final pH attained from growth in the presence of the readily fermentable carbohydrates ranged from 4.2 to 4.4. Acetyl methyl carbinol was formed in the glucose fermentation.

Optimum growth occurred at 37° C., but the organism grew between 25° and 45° C. and survived heating at 60° C. for 30 minutes.

The strain was not typable by any of the streptococcal major group antisera. It does not appear to resemble closely any of the streptococci described in the latest edition of *Bergey's Manual.*[7]

A 48-hour thioglycollate broth culture of this organism was introduced into the germ-free unit in two sealed ampoules through a germicide trap[8] for the purpose of

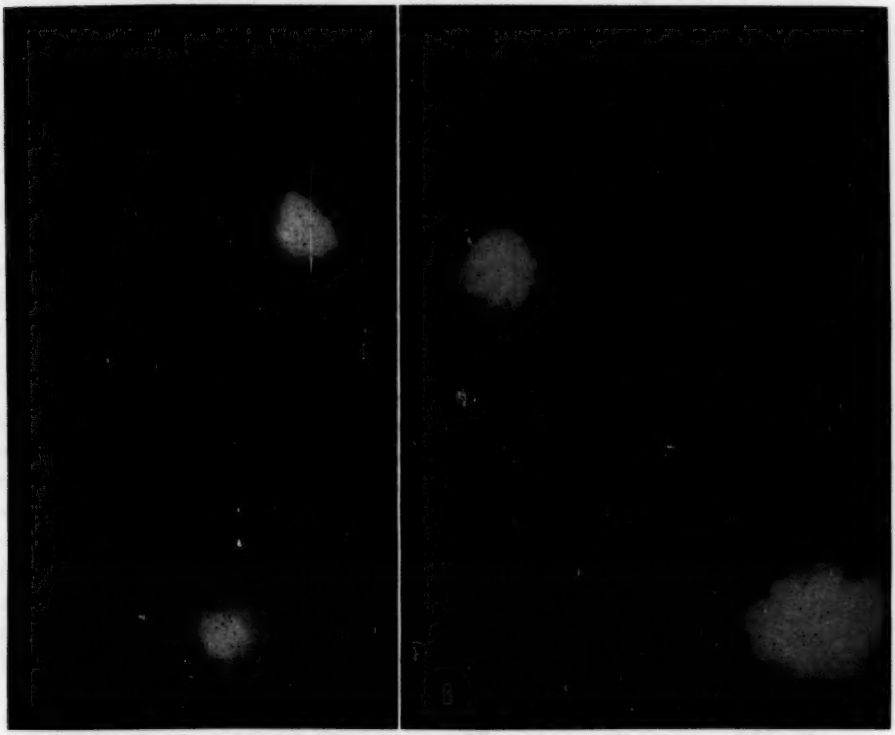

FIG. 1.—*A*, surface colony of rat oral streptococcus on trypticase-soy agar. (Mag. ×22.) *B*, subsurface colony of the same organism. (Mag. ×22.)

establishing the culture. One of the ampoules was opened within the apparatus, and the culture was inoculated into the oral cavity of each animal by means of a small cotton swab. The food cups and drinking fonts were also inoculated in this fashion. The second ampoule following removal from the apparatus, was used to treat the conventional control animals in a similar fashion and also provided a check on the survival of the inoculum during the germicidal-trap passage.

Weekly inspections were made, using a variety of bacteriological culture media, to insure that the isolator units were not contaminated by any extraneous micro-organisms. Stained smears and cultures of the feces and oral cavity of the inoculated group and germ-free group were also routinely made.

At the termination of the experiment the animals were autopsied under aseptic conditions, and samples of blood, tissues, feces, and cecal contents were cultured to confirm the absence of contaminating micro-organisms. The heads were removed and the skulls split sagittally. Half was stripped of tissues for caries diagnosis, and the other half was fixed in neutral formalin for histologic study. Representative organs were similarly preserved. Tissue sections were stained by hemotoxylin and eosin and the Brown and Brenn modification of the Gram stain.

RESULTS

The effects of autoclaving the diet on the growth rates of conventional, inoculated, and germ-free animals are illustrated in Figure 2. A growth curve is also included for a group of conventional rats receiving the non-autoclaved diet. Although the animals maintained on the autoclaved diets were obviously undersized, they appeared normal and healthy in other respects except for a transient slight diarrhea during the first 2 weeks on the diet.

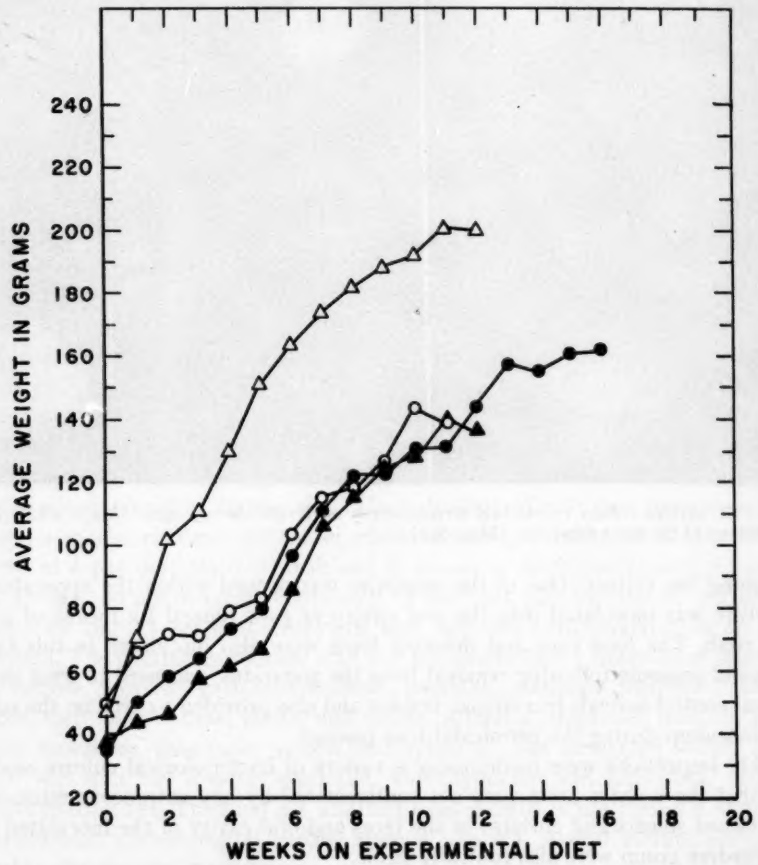

FIG. 2.—Effect of diet sterilization on growth of germ-free and inoculated rats; ● germ-free control rats; ○ inoculated rats; ▲ rats on conventional autoclaved diet; △ rats on conventional unautoclaved diet.

Caries.—Each of the eight rats in the conventional control group, sacrificed after 100 days on experiment, and all ten animals in the inoculated group, sacrificed after 77 days, showed extensive caries of the molar sulci, especially in the first and second molars (Fig. 3, *B* and *C*). In contrast, the teeth of the germ-free animals, sacrificed after 84 days, were free of caries in spite of considerable food impaction and the occasional fracture of a molar cusp (Fig. 3, *A*). Decalcified sections of the germ-free molars showed no abnormalities (Fig. 4, *A*), while the appearance of the inoculated animals was typically that of far-advanced cavitation involving the dentin and occasionally penetrating the pulp chamber, resulting in intrapulpal abscesses (Fig. 4, *B*).

Histopathology.—Except for the characteristically enlarged cecum in the germ-free and inoculated animals, there were no significant abnormalities in any of the extra-oral tissues examined. Within the oral cavity all pathologic changes were confined to the molar regions.

In the germ-free controls group three maxillary quadrants were studied in detail. No caries or micro-organisms were found. However, all interproximal areas showed ulceration of the gingival papillae and leukocytic exudation, resulting apparently from irritation by imbedded hairs (Figs. 5–7). In some cases (Fig. 6) the profuse exudate filled the interproximal space. Figure 7, a higher magnification of this area in Figure 6, contains an irregular dark area which, in structure and staining characteristics with the hematoxylin-eosin and Brown and Brenn stains, resembled decalcified calculus.

From the inoculated group, one mandibular and four maxillary quadrants were studied in detail. Only one molar, a maxillary third, was not carious. All except two of the interproximal areas contained imbedded hairs with the attendant inflammatory response. In four of the molars, caries was severe enough, despite the deposition of secondary dentin, to involve the pulp tissue with the formation of intrapulpal abscesses (Fig. 4, *B*). Despite the presence of large numbers of micro-organisms, no infection of the approximating gingival tissues was apparent. A deeper section of the same interproximal space as that shown in Figure 4, *B*, revealed replacement of the papillary structure with a crater-shaped lesion lined by stratified squamous epithelium without rete pegs (Figs. 8, 9).

A typical foreign-body, giant-cell reaction to an imbedded hair is shown in Figure 10. There was, however, no invasion of the inflamed area by the micro-organisms used to inoculate the animal, despite active caries in the occlusal sulci of the adjacent teeth.

Except for the differences in microbial flora, the oral tissues of the inoculated animals presented much the same appearance as did those of the conventional controls. Figure 11 shows a section of an interproximal area in a conventional rat in which ulceration of the gingival papilla around an impacted hair resulted in a pronounced leukocytic exudation encompassing hair, food, and cellular detritus in a "plaque." Inflammation and organisms were confined to the papilla without involvement of the adjacent tissues, thus resembling the condition observed in the inoculated animals.

DISCUSSION

The production of caries in gnotobiotic rats as reported by Orland *et al.*,[2] was accomplished in animals carrying an enterococcus plus one other micro-organism. Later, Orland[9] demonstrated caries with a pure culture of this same enterococcus. It was also pointed out that possibly other types of micro-organisms would be found capable of producing caries in the gnotobiotic animal.

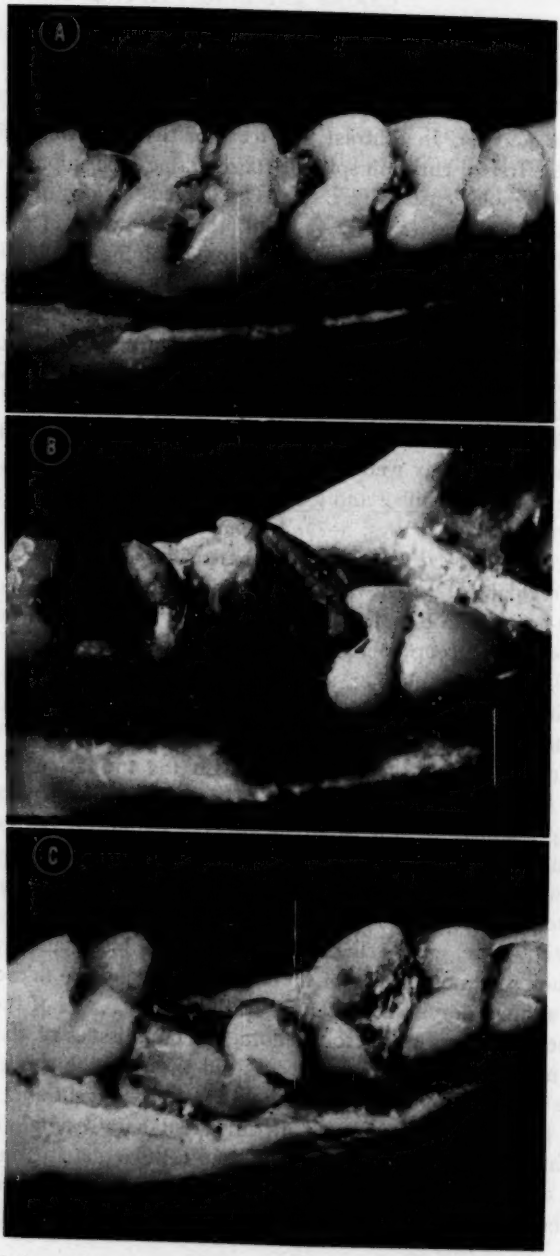

FIG. 3.—*A*, mandibular molars of germ-free control rat, showing impacted food particles and slight fracturing of the cusps in the first and second molars. *B*, mandibular molars of inoculated rat, showing extensive caries in the first and second molars and fracturing of the third molar. *C*, mandibular molars of conventional control rat, showing caries and fracturing in first and second molars.

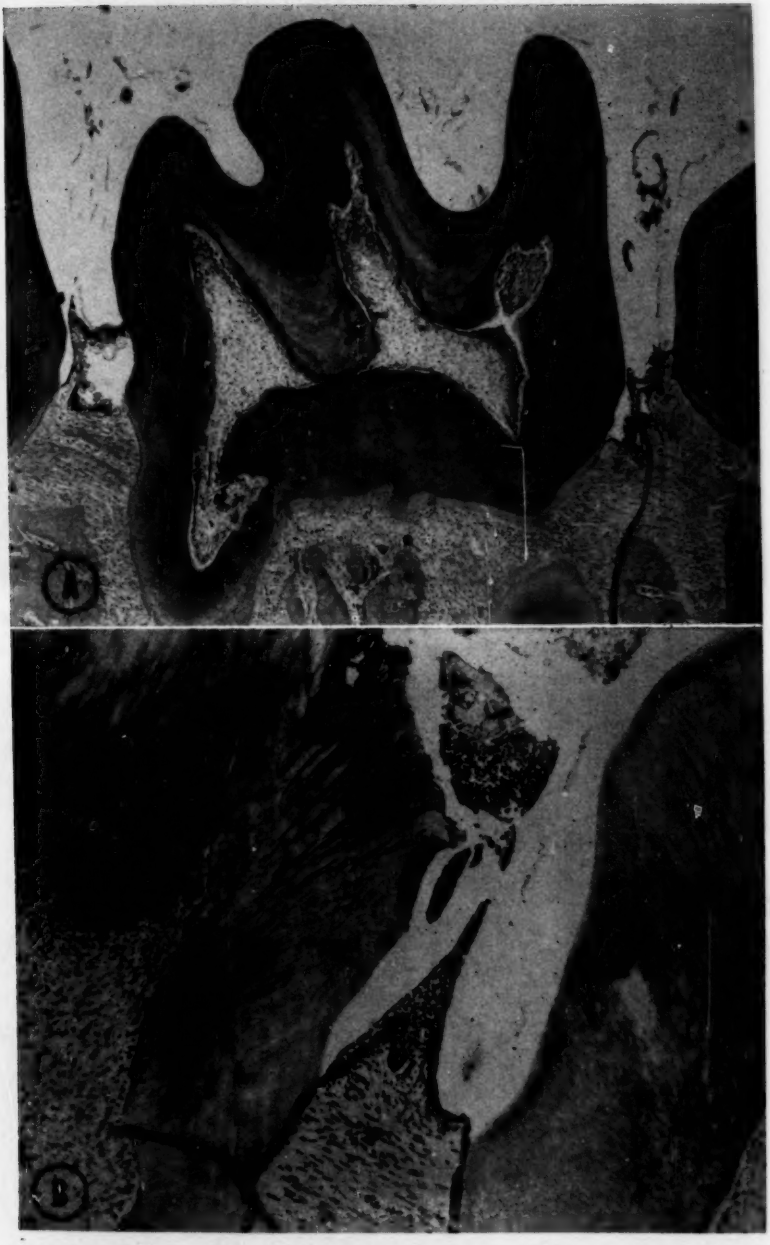

FIG. 4.—*A*, decalcified *second* mandibular molar of germ-free control rat. (Brown and Brenn stain; mag. ×40.) *B*, decalcified section of inoculated rat, distal of second and mesial of third molar, showing carious exposure of pulp cavity and secondary dentin formation. (Brown and Brenn stain; mag. ×115.)

FIG. 5.—Second maxillary molar, germ-free control, showing impacted hairs in the interproximal areas.

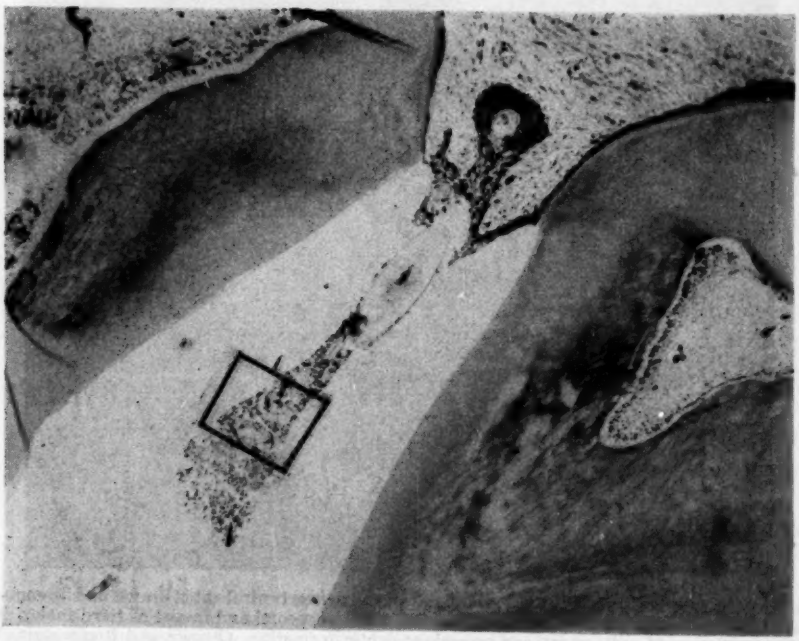

FIG. 6.—Decalcified section, germ-free control, distal of second and mesial of third maxillary molars, showing inflammatory response to hair impacted in the gingival papilla. (Brown and Brenn stain; mag. ×70.)

FIG. 7.—Higher magnification of area outlined in Fig. 6, showing focus of possible calcified area. (Brown and Brenn stain; mag. ×165.)

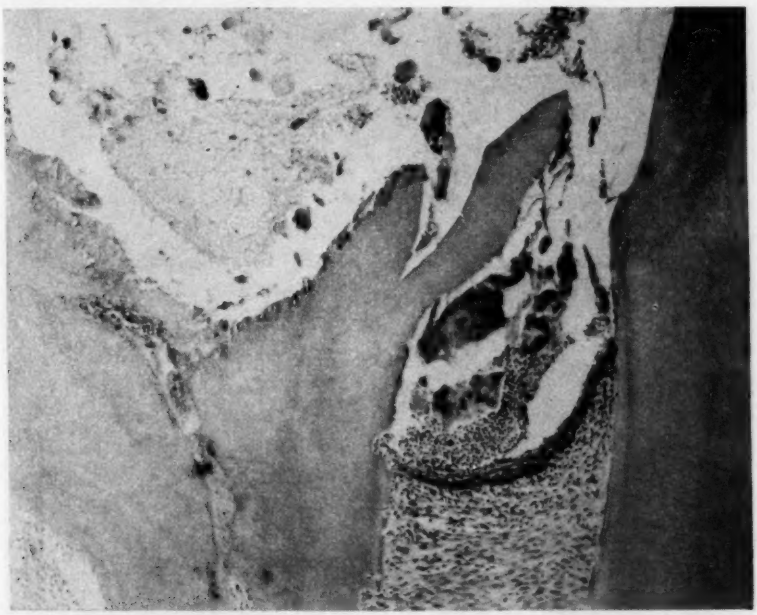

FIG. 8.—Inoculated animal; deeper sections of same area shown in Fig. 4, showing replacement of papillary structure with crater-shaped lesion lined by stratified squamous epithelium and extensive dentinal caries extending into pulp chamber. (Hematoxylin and eosin stain; mag. ×70.)

FIG. 9.—Same area as shown in Fig. 8; showing bacterial involvement of pulp and microbial plaque in close proximity to gingival lesion. (Brown and Brenn stain; mag. ×70.)

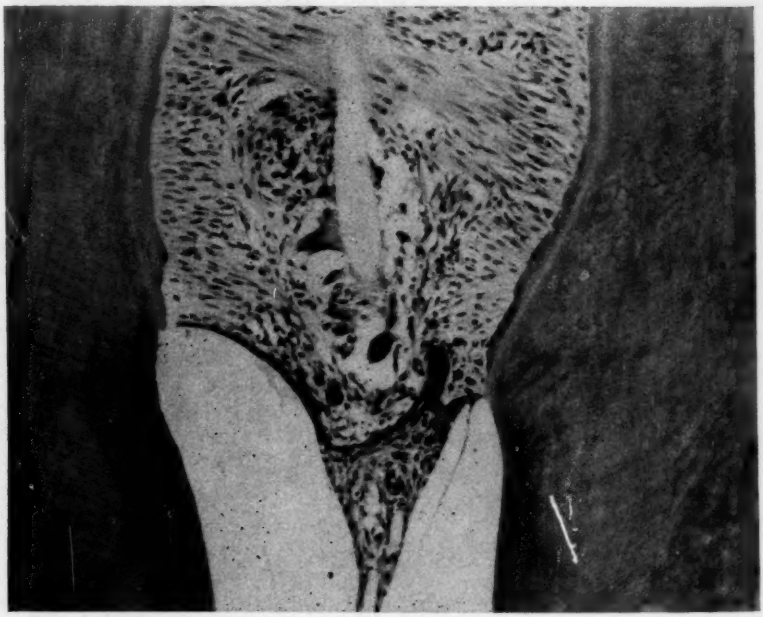

FIG. 10.—Inoculated animal, distal of first and mesial of second maxillary molars, showing typical foreign-body, giant-cell reaction to an imbedded hair. (Hematoxylin and eosin stain; mag. ×235.)

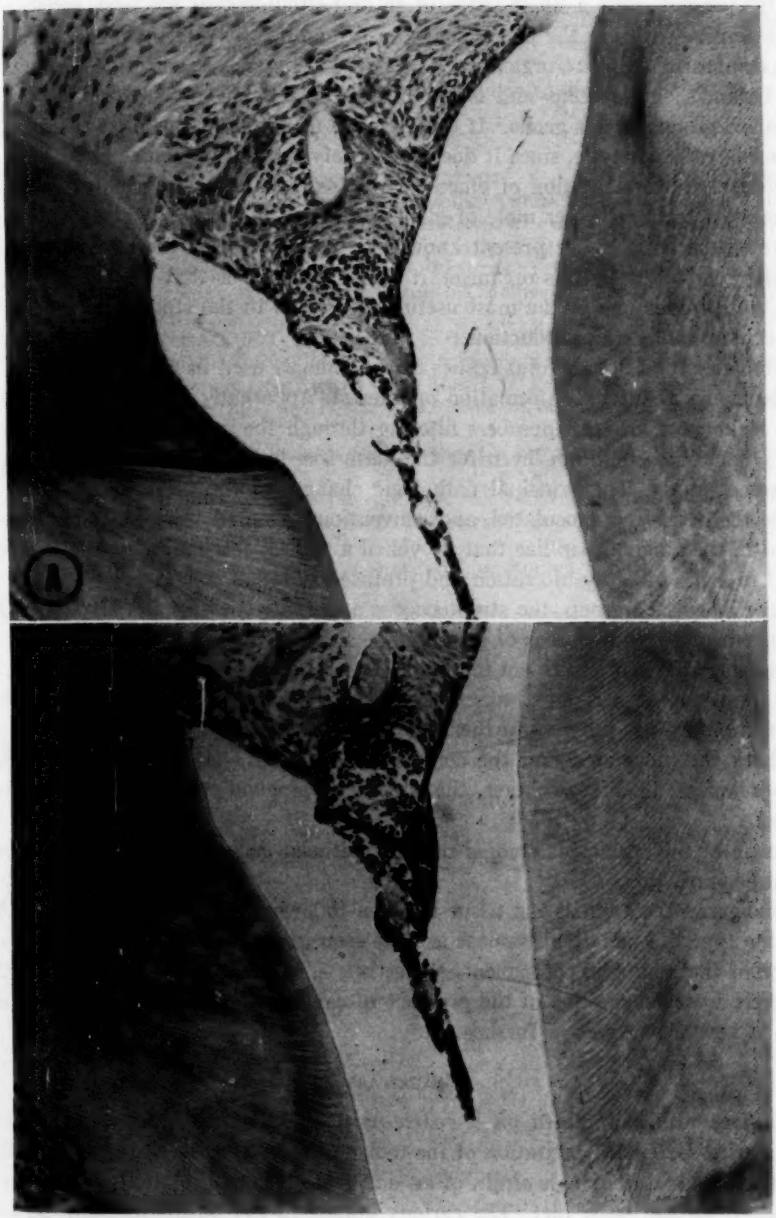

FIG. 11.—*A*, conventional animal, distal of first and mesial of second maxillary molars, showing ulceration of gingival papilla around an imbedded hair. (Hematoxylin and eosin stain; mag. ×165.) *B*, same area shown in *A*. (Brown and Brenn stain; mag. ×165.)

In the present study a single strain of an oral streptococcus has been shown to produce extensive caries in the molar dentition of gnotobiotic rats in the absence of any other demonstrable micro-organism. The active streptococcus is of a different type from Orland's enterococcus and is not readily classifiable into any of the currently recognized groups of this genus.[7] It is of interest to note that this organism would not be considered proteolytic, since it does not liquefy gelatin, peptonize milk, or produce collagenase. Its fermentation of glucose is typically homolactic, the final yield being 1.8 moles of lactic acid per mole of glucose utilized in vitro. While it would be premature on the basis of our present knowledge to speculate on the in vivo mechanism of caries production by this organism, it should be obvious that the gnotobiotic technique will provide one of the most useful approaches to the study of the problem of the mechanism of caries production.

Histologic studies of the oral tissues of the animals used in the present study have revealed some additional information of interest. Apparently, the presence of neutrophilic leukocytes in small numbers filtering through the crevicular epithelium represents a "normal" condition, even for the germ-free animals, under the conditions of the present study. The principal pathologic changes of the periodontal tissues common to the germ-free, inoculated, and conventional animals were related to hairs imbedded in the gingival papillae that provoked a typical foreign-body reaction, accompanied in some cases by ulceration and profuse exudation.

In the inoculated group, the streptococcus apparently possessed a limited degree of virulence because, in the areas of ulceration accompanying impacted hairs, the approximating gingival tissues were not involved. Even when caries was so severe as to result in the formation of intrapulpal abscesses with extension to the periodontal membrane, the infection did not progress further.

In both the inoculated and the conventional animals, despite massive growths of micro-organisms in direct contact, the superficial gingival tissues were generally unaffected.

In none of the specimens studied did the epithelial attachments migrate below the cemento-enamel junction.

In the germ-free animals the tissue reaction following the impaction of hairs led to the same type of exudative response as that seen in the other groups, except for the absence of the microbial component. Nevertheless, in at least one instance, the histologic picture was suggestive of the presence of calcification in the plaque. This observation is now the subject of further study.

SUMMARY

Germ-free rats maintained on a coarse-particle, high-sugar diet did not develop dental caries. Extensive cavitation of the molar teeth was observed in gnotobiotic animals inoculated with a single strain of an oral streptococcus isolated from the rat and maintained on the same diet. The carious lesions in the inoculated animals closely resembled those seen in conventional (non-germ-free) rats receiving the same diet. Histologic studies of the oral tissues of the germ-free animals revealed typical foreign-body reactions and ulceration associated with hairs imbedded in the gingival papillae. In the inoculated animals, infected intrapulpal abscesses resulted from the extensive carious process, but the superficial gingival tissues did not become infected.

We are indebted to the Lobund Institute of the University of Notre Dame and, in particular, to Professor P. C. Trexler for supplying the animals used in this study. The co-operation of Mr. J. L. Hickey, chief of the Germfree Services Section, NIH, is gratefully acknowledged. Dr. S. E. Mergenhagen performed the collagenase and chondroitinase tests. Dr. T. A. Nevin rechecked many of the biochemical tests, and Dr. R. M. Cole provided the information on the serological tests.

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Further Studies into the Vascular Architecture of the Mandible

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In previous papers[1,2] the methods of investigating the vascular architecture of the mandible were described. This paper deals with the detailed histologic examination of sections of cat mandibles injected with warm carmine gelatin via one common carotid artery.

EXPERIMENTAL METHODS

As warm carmine gelatin has a particle size of approximately 1 μ, the injection material will traverse the capillaries. Thus the injection material passes from the arterioles to the capillaries and then into the veins. The method of preparation of the sections has been described elsewhere.[2] Celloidin sections were cut in a coronal plane at 200 μ and in a sagittal plane at 100 μ, cleared, and mounted.

RESULTS

The mandible is covered by a layer of periosteum over the whole of its surface. Imbedded within the mandible are the teeth, which are attached to the wall of the tooth socket by the periodontal membrane. The oral aspect of the mandible is clothed with mucous membrane. Each of these different structures—periosteum, bone, periodontal membrane, tooth, and mucous membrane—has a blood supply. In an investigation into the vascular architecture of the mandible the blood supply of these structures must be considered.

Periosteum.—Where muscles and tendons are attached to the mandible, the attachment of the periosteum to the bone is firm. This close connection depends mainly on the continuation of dense collagenous bundles from the periosteum into the bone as Sharpey's fibers. At such places, blood vessels and nerves enter the bone (Figs. 1 and 2). Where the periosteum is loosely connected, there are only a few thin collagenous bundles, and attachment to the bone is maintained largely by small blood vessels.

The author believes that the periosteal blood supply contributes mainly to the supply of the lower border of the mandible. Numerous blood vessels are seen entering the bone from the periosteum at the lower border of the mandible (Figs. 1 and 2).

Bone.—In the human mandible the marrow spaces contain fatty marrow. Hemopoietic cellular marrow is found only in the condyloid process and in the region of the angle.[3] In the cat, microscopic examination of coronal and sagittal sections of the

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mandible show the marrow spaces to contain fatty marrow; scattered between the fat are small blood vessels of capillary size. No sinusoids have been observed in the marrow spaces. There is a well-marked capillary network in the marrow spaces of the mandible (Figs. 1–5), and through this capillary network the arterial and venous sides of the circulation communicate.

Coronal sections of the cat mandible (Fig. 1) show that the inferior dental artery supplies branches to a large vessel at the junction of the cancellous and cortical bone.

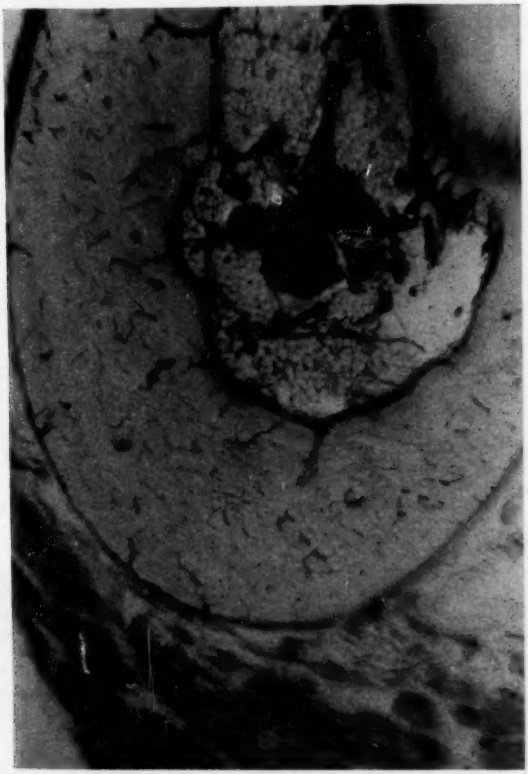

FIG. 1.—Coronal section of the mandible. An arterial circle may be seen as the junction of cortical and cancellous bone from which branches are given off to the cortex. Numerous perforating vessels may be seen at the lower border of the mandible. (Mag. ×30.)

From this vessel branches are derived which penetrate as far as the middle of the cortex but no farther. The inferior dental artery of each side is the principal nutrient artery. An anastomosis exists between the inferior dental artery and the periosteal system of blood vessels (Figs. 1 and 2).

The caliber of the vessels in bone is greater toward the alveolar border than toward the lower border (Fig. 1). Thus it may be concluded that the lower border of the mandible has a poorer blood supply than the alveolar portion of the bone.

Periodontal membrane.—The blood supply of the periodontal membrane, as shown in the present investigation, is in agreement with that described by Scott and Symons.[4]

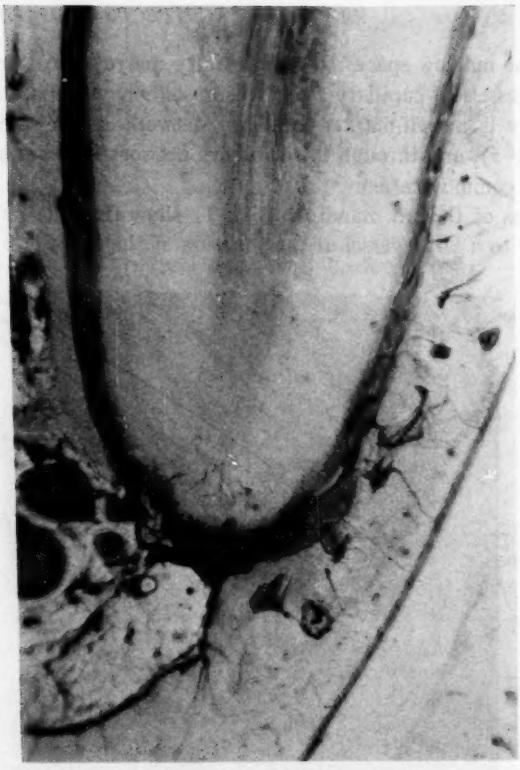

FIG. 2.—Coronal section of the mandible, showing the blood supply of the periodontal membrane. (Mag. ×30.)

FIG. 3.—Apex of a tooth with vessels passing through the apical foramina into the pulp. Perforating branches can be seen passing through the lamina dura to supply the periodontal membrane. (Sagittal section; mag. ×30.)

It is derived from three sources: (1) From the apical region of the tooth. As they traverse the periapical tissue, the vessels to and from the pulp give off and receive vessels passing to and from the periodontal membrane (Fig. 3). These periodontal vessels pass between the bundles of the principal fibers in their passage to and from the gums. Some of the vessels lie close to the wall of the socket in grooves and are therefore protected from the effects of pressure. (2) From the alveolar bone. Branches perforate the lamina dura and form anastomoses between the main periodontal vessels and the vessels of the Haversian systems and cancellous tissue of the alveolar bone (Fig. 3). (3) From the gingival tissue. These branches anastomose with the main periodontal vessels around the necks of the teeth (Figs. 6 and 7).

Pulp.—The pulp is contained within the pulp chamber and root canal or canals of

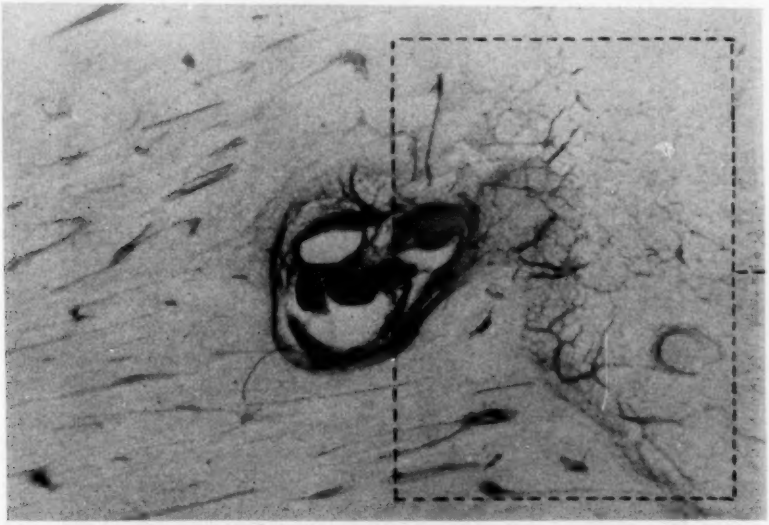

FIG. 4.—Injected blood vessels in bone. Note the capillaries of the marrow spaces emptying into veins at the junction of cortical and cancellous bone. Fig. 5 is an enlarged view of the enclosed area on the right. (Sagittal section; mag. ×30.)

the tooth. It is highly vascular (Fig. 8), and its blood vessels enter through the apical foramen. Usually one artery and one or two veins occupy each foramen. The artery carrying the blood into the pulp branches out into a rich network of blood vessels soon after entering the root canal. The veins gather blood from this capillary network and carry it back through the apical foramen into larger vessels. The arteries are clearly identified by their straight course and thicker walls, whereas the thin-walled veins are wide and frequently have an outline similar to a row of beads (Fig. 8). The capillaries form loops close to the odontoblasts near the surface of the pulp and may even reach into the odontoblast layer.[3]

Blood vessels entering and leaving the pulp via the apical foramina and their course within the pulp have been demonstrated (Figs. 3, 8, and 9).

Mucous membrane.—Dissection and histologic examination have shown that the facial and lingual arteries supply the mucous membrane. The interdental vessels send

branches into the mucous membrane (Figs. 6 and 10). The vessels within the periodontal membrane anastomose with those of the gingivae around the necks of the teeth (Figs. 6 and 7).

The arteries.—In this investigation it has been found that the arteries are more easily filled than the veins. The arterioles of the marrow throughout their course undergo little or no decrease in caliber, and their branches are approximately the same size as themselves; in their passage through the marrow they pursue a straight course, changing direction by sudden angulations. This characteristic has also been found to apply in situations other than the marrow, e.g., periodontal membrane (Figs. 2 and 7).

The veins.—The venous radicles coming from capillaries within the fatty marrow (Fig. 5) are broader and run a more irregular course than the equivalent arteries.

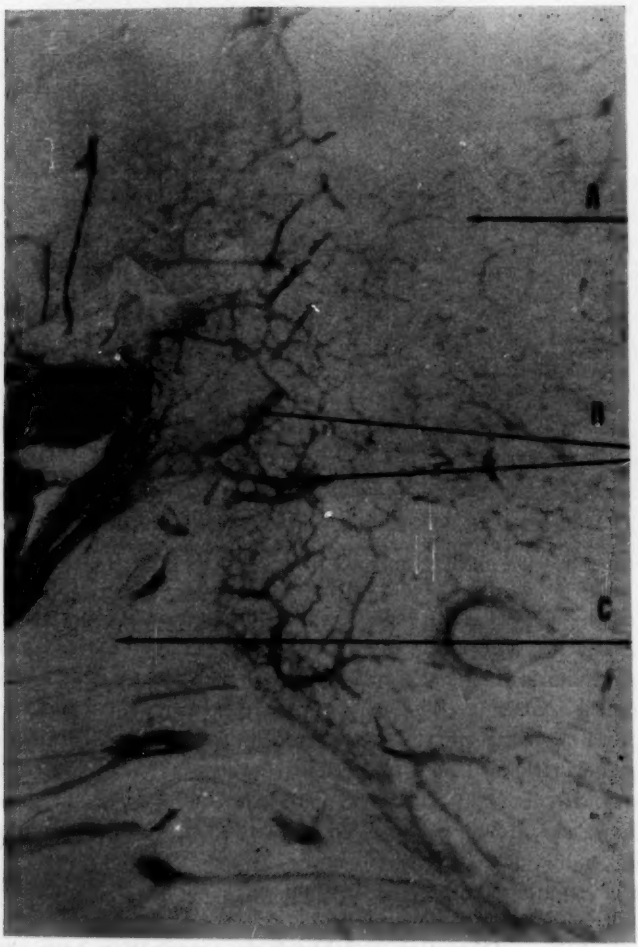

FIG. 5.—Enlarged view of Fig. 4. *A* = cancellous bone; *B* = venules; *C* = cortical bone. (Sagittal section; mag. ×60.)

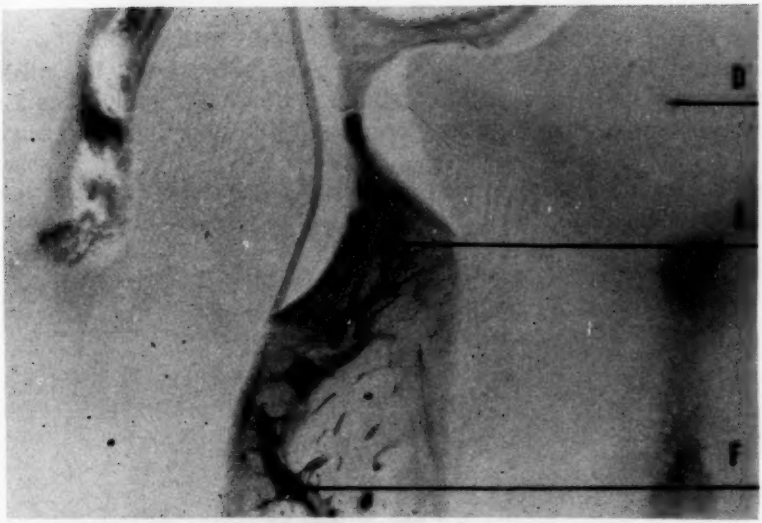

FIG. 6.—Interdental vessel piercing the lamina dura and supplying the gingivae. D = dentin; E = gingivae; F = interdental blood vessel. (Sagittal section; mag. ×30.)

FIG. 7.—Interradicular vessel passing through the lamina dura and supplying a branch to the periodontal membrane. D = dentin; E = gingiva; G = periodontal blood vessel passing into gingiva; H = blood vessel in periodontal membrane. (Sagittal section; mag. ×30.)

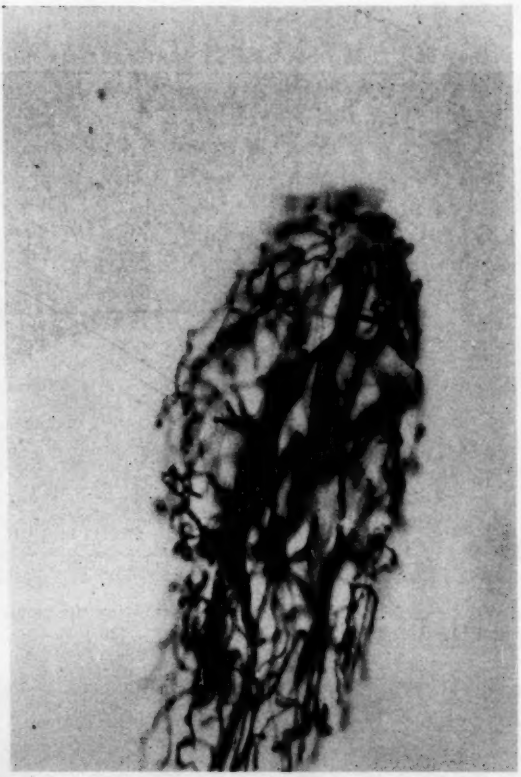

FIG. 8.—Blood vessels of the pulp. India ink–injected specimen. (Mag. ×400.)

FIG. 9.—Longitudinal blood vessels in the pulp. Injected blood vessels in the interdental septum and periodontal membrane are well shown. (Sagittal section; mag. ×30.)

DISCUSSION

In the past there has been little research into the vascular architecture of the mandible. Keller and Cohen[5] demonstrated the vascular plexus of the oral tissues in dogs. Schuback and Goldman[6] studied the vascularity of the gingivae of the dog. Perint[7] investigated the blood supply of the teeth and jaws, using material from the cat, dog, and human.

A great deal of investigation has been carried out on the blood supply of the long bones. Some of the findings are relevant to the discussion of the vascular architecture

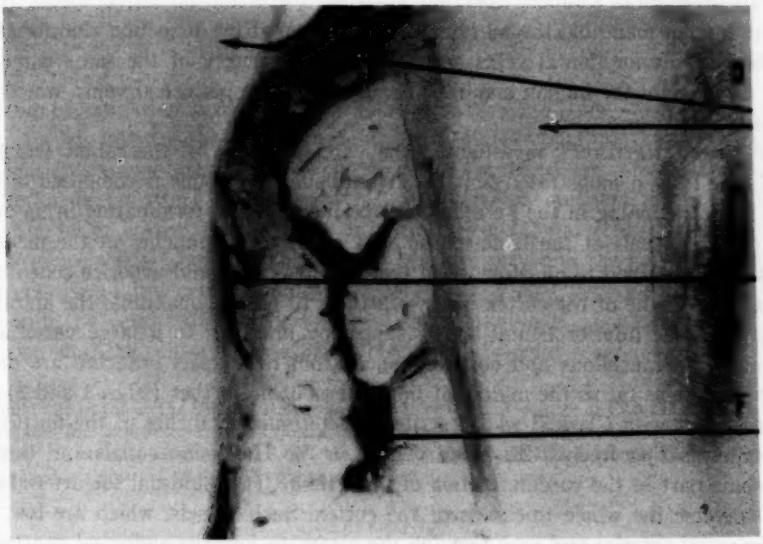

FIG. 10.—Interdental vessel giving branches to the periodontal membrane of the tooth on either side and supplying branches which terminate in the gingival margin. *D* = dentin; *F* = interdental blood vessel; *H* = blood vessel in periodontal membrane. (Sagittal section; mag. ×30.)

of the mandible. In the mandible the inferior dental artery of each side may be compared with the nutrient artery of a long bone. How much of the blood supply of the mandible is contributed by the inferior dental arteries, and how much by the arteries of the periosteum?

Much discussion in the past has centered on the respective contributions which the periosteal arterial network and the principal nutrient artery make to the vascularization of a long bone. The earlier authors, e.g., Barkow,[8] Langer,[9] and Testut,[10] considered the periosteal vessels to be of prime importance. Recently this has been disputed. Drinker, Drinker, and Lund,[11] in injection experiments, considered the periosteal vessels to be of no importance, while Johnson[12] thought them to be of minor importance. Other authors who support them in their views that the principal nutrient artery is pre-eminent in the vascularization of a long bone are Harris,[13] Kistler,[14] Wood-Jones,[15] Marneffe,[16] Watson-Jones,[17] and Laing.[18] Gray,[19] Testut and Latarjet,[20] Cunningham,[21] and Ham[22] state that there is a free anastomosis between the principal nutrient artery and the periosteal system. The author has demonstrated in the cat mandible a

free anastomosis between the inferior dental artery, the principal nutrient artery of the mandible, and the periosteal system (Fig. 1).

The venous drainage of a bone has attracted little attention. Langer[9] first observed that there are more veins leaving bone than there are arterial twigs entering. Lamas, Amado, and Da Costa[23] stated that large "venous lakes" are present in cancellous bone at the extremities of long bones, while Marneffe[6] described a central venous channel extending the length of the medulla. The author[2] has demonstrated a single venous channel in the cat mandible. Other authors who have observed a central medullary vein are Rustizky,[24] who noted its presence in frogs, and Bizzozero,[25] who recorded its presence in a rabbit tibia.

In the human mandible the author has demonstrated by dissection the presence of a number of inferior dental veins accompanying the artery of the same name. The venous drainage of the human mandible is mainly via the periosteal veins, which drain into the jugular veins.[1]

Brookes and Harrison[26] investigated the vascularization of the rabbit femur and tibio-fibula. They demonstrated that the arterial supply of bone is composed of longitudinal vessels running in the periphery of the medulla and terminating in an arborization of fine vessels at the bone extremities. Transverse branches of the medullary arteries were observed to anastomose in the endosteal zone and arborize centrifugally as far as the middle of the cortex but no farther. In the cat mandible the author has demonstrated the inferior dental artery supplying branches to a large vessel at the junction of the cancellous and cortical bone. From this vessel branches are derived which penetrate as far as the middle of the cortex but no farther (Figs. 1 and 2). This is in agreement with Clark,[27] who describes the terminal branches of the nutrient artery communicating freely with blood vessels in the Haversian canals and therefore taking some part in the vascularization of bone tissue. It is unusual for arterial channels to traverse the whole thickness of the cortex. Such vessels, which are few, arise from periosteal arteries and pass through the cortex to join the endosteal anastomotic network, thereby augmenting the medullary arterial system derived from the branchings of the principal nutrient artery and their anastomoses at either end of the bone with the nutrient arteries entering there. In particular, the cortex of the shaft of the long bone does not depend on periosteal twigs for nutrition.

Brookes and Harrison[26] showed that the medullary venous system is composed of sinusoids which drain into a central venous channel and its radiating branches. The author has been unable to demonstrate the presence of sinusoids in the cat mandible.

There is much controversy about the blood vessels of the cortex. Langer[9] claimed that at least an artery and vein can be found in a single canal and often a leash of vessels of capillary size. Testut and Latarjet[20] consider the vessels to be capillaries, yet nevertheless speak of arterioles entering the cortex from the periosteal network and also of medullary venous capillaries being in continuity with venules in larger Haversian canals. Lacroix[28] states that there are one or two capillaries in a Haversian canal, while Marneffe[16] describes but a single endothelial tube surrounded by a slight adventitia in Volkmann's canals. Maximow and Bloom[29] state that a Haversian canal carries one or more, usually two, blood vessels which are capillaries or postcapillary venules. Occasionally an arteriole is found in a canal.

It is commonly accepted that arteries enter the cortex from both its periosteal and

its endosteal surface and anastomose with one another in the canal system, the same applying to the veins. Brookes and Harrison[26] found that in the rabbit there is a vascular lattice in the cortex composed entirely of simple endothelial tubes which is in continuity with the medullary venous sinusoids, on the one hand, and the osteogenic capillaries, on the other. Into this lattice empty the terminal arborizations of the medullary arteries. They were unable to decide whether these endothelial tubes were arterioles or precapillaries.

Although simple endothelial tubes in the cortex of the rabbit femur and tibio-fibula may connect the medullary sinusoids with the osteogenic capillaries,[26] this is not so in the cat mandible. In the latter an extensive capillary network connects the arteries with the veins.

SUMMARY

In this investigation warm carmine gelatin was injected via one common carotid artery, to demonstrate the vascular architecture of the mandible in adult cats. The histologic appearance of sections of cat mandibles is described. The author considers the inferior dental artery of each side to be the principal nutrient artery of the mandible. The lower border of the mandible is supplied by the arteries of the periosteum. An anastomosis exists between the inferior dental artery and the periosteal system of blood vessels. In the cat mandible an extensive capillary network connects the arteries with the veins. The blood supply of the mandible is compared with that of a long bone, and the relevant literature is discussed.

I should like to thank the Clinical Research Committee of the Institute of Clinical Research, Middlesex Hospital, for facilities granted in the Institute. My thanks are also due to W. Warwick James for suggesting the investigation and to E. W. Walls, J. W. Schofield, and D. Greer Walker for criticism and advice. I am grateful to R. A. Kimber for his technical assistance.

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Effect of Some Vasoconstrictors on Intradermal Anesthesia

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Although Braun[1] and earlier workers showed more than 50 years ago that the addition of epinephrine to local anesthetic solutions increased the duration of anesthesia, few investigations have been carried out on the quantitative aspects of the potentiation induced by vasoconstrictors.

In this investigation, the effect of graded concentrations of epinephrine, levarterenol, and levo-nordefrin on the anesthesia induced by procaine and propoxycaine has been studied.

EXPERIMENTAL METHODS

The technique of Bülbring and Wajda[2] was used in all experiments. Local anesthetic activity was determined by the intradermal injection of dilutions of the original solution graded at 0.15 log intervals (Dilution Test). Two wheals on the back of each guinea pig were raised, one with the experimental and the other with the control solution. Eight or more guinea pigs were injected with each solution. Readings were taken every 5 minutes for 30 minutes. The mean sum of scores (maximum = 36) was plotted against the dilution on semilog paper, and the dilution, producing a mean score of 5 (Threshold Anesthetic Dilution 5 or TAD_5), was estimated from the dose-effect curve.[3]

To determine duration of intradermal anesthesia, readings were taken every 10 minutes, and the mean score for each reading (maximum score = 6) was plotted against time. The mean duration represents the time in minutes at which the mean score reached 50 per cent of maximum anesthesia (score = 3) as determined from the mean score–time curves by interpolation.[4]

The mean duration determined by this method ($mean_1$), which is used preferentially in this study and is shown in all tables, is not identical with the mean of the durations of anesthesia of the individual wheals ($mean_2$), although the difference in the experiments in which both means were calculated did not exceed 15 per cent.*

Of the twenty-two $mean_2$ values shown in Tables 4, 5, and 6, seven were approximations. In one of these seven determinations, three of the wheals and, in the other six, one of the wheals had a local anesthetic score higher than 3 at the end of the experiment. In order to compute the $mean_2$ values, it was assumed that the local anesthetic effect would have ended at the next reading. Therefore, the $mean_2 \pm$ s.e. in

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* $Mean_1 = 100$. In the twenty-two experiments in which the two means were calculated, there was no difference in two. In ten experiments $mean_1$ was higher than $mean_2$ (the average difference being 3.1 per cent), and in the other ten, $mean_2$ was higher than $mean_1$ (average difference = 3.3 per cent).

those experiments should have been somewhat higher. The results would not have been significantly changed if the values from the wheals which had not recovered had not been included in the computation of the $mean_2$ values or if the anesthesia in these wheals had been assumed to last 100 minutes beyond the end of the experiment. In other words, the P values in Tables 4, 5, and 6, which were > 0.1 and < 0.01, would have remained > 0.1 and < 0.01, respectively.

The relationship between the *mean score*–time curves and the score-time curves of the individual wheals in one of the experiments is shown in Figure 1. In 78 per cent of the wheals that recovered completely, the decline from maximum to zero anesthesia was relatively rapid (less than 30 minutes).

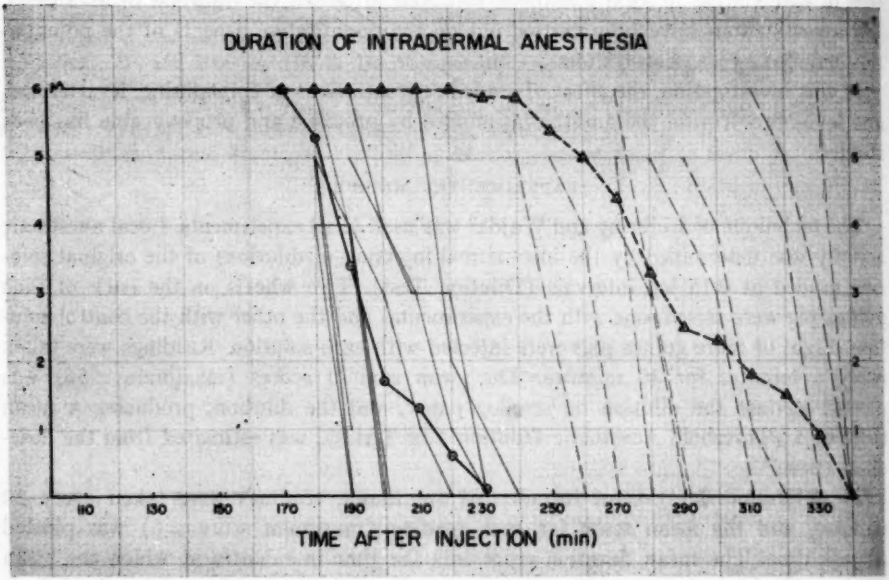

FIG. 1.—Parallel test with solutions *A* and *B* on ten guinea pigs. (Solution *A* = propoxycaine HCl: 0.25 per cent, procaine HCl; 2.0 per cent; and epinephrine HCl: 1:100,000 in terms of base; solution *B* = propoxycaine HCI 0.25 per cent and epinephrine HCI: 1:100,000 in terms of base.) *Ordinates:* Local anesthetic scores (Bülbring and Wajda's technique). *Solid lines* = solution *A; broken lines* = solution *B*. *Thin lines* = individual score-time curves; *thick lines* pass through the points obtained by plotting the mean score at each reading against time.

In the experiments in which the $mean_2$ was determined, the standard error was within 15 per cent of this value.

In both the dilution and the duration tests, each of the mixed solutions was tested in parallel with a control solution. With the exception of the concentration-duration regression lines obtained by plotting the mean duration against the concentration of the vasoconstrictor in 0.25 per cent propoxycaine solutions, the relative duration (control = 100) has been preferred to the actual mean durations in interpreting the results.

The drugs used were procaine HCl, propoxycaine HCl* (2-diethylaminoethyl-4-

* Ravocaine(R) HCl; available from Cook-Waite Labs., Inc.; and Blockain(R), George A. Breon and Company.

amino-2-propoxy benzoate HCl), epinephrine bitartrate (suprarenin bitartrate), levo-arterenol dextrobitartrate monohydrate, and levonordefrin* (base) (levo 2,4-di-hydroxy-phenyl-3-hydroxy-2-isopropylamine). The concentration of the local anesthetics was expressed in terms of the hydrochloride salts; the vasoconstrictors' in terms of the bases.

RESULTS

A solution containing 1:100,000 epinephrine in saline was injected intradermally on the back of 10 guinea pigs. The other wheal was raised with a solution of 0.5 per cent procaine HCl + epinephrine 1:100,000. Some anesthesia was produced by the epinephrine solution, but it was incomplete, the highest average score (reached 40 minutes after injection) being 2.5. The other solution produced complete anesthesia (score = 6) for more than 160 minutes.

TABLE 1

INTRADERMAL ANESTHESIA: DILUTION TESTS

(Guinea Pigs)

Propoxycaine HCl 0.25% + Epinephrine — Conc. of Epinephrine	Local Anesthetic Activity			Concentration of Propoxycaine HCl (P) and Epinephrine (E) at TAD_5	
	Propox. HCl + Epineph.		Control*		
	TAD_5	Per Cent (Control = 100)		P†	E
1: 25,000.....	1/54	470	1/11.2	0.0046	1:1,350,000
1: 50,000.....	1/34.5	310	1/11.	0.0072	1:1,720,000
1:100,000.....	1/25	210	1/11.7	0.010	1:2,500,000
1:200,000.....	1/23.3	200	1/11.7	0.011	1:4,660,000
1:400,000.....	1/24.5	177	1/13.8	0.010	1:9,800,000

* Propoxycaine HCl 0.25 per cent sol. *without* epinephrine.

† The average concentration of propoxycaine HCl at the TAD_5 of the control solutions was 0.021%. In all the concentrations tested, epinephrine increased the potency of propoxycaine. With the largest concentration used, the increase was almost fivefold.

A solution of 0.25 per cent propoxycaine (control solution) was tested in parallel with graded concentrations of epinephrine, ranging from 1:25,000 to 1:400,000. The results (Table 1) show that the increase in activity was proportional to the concentration of epinphrine only with the three highest concentrations.

As shown in Table 2, epinephrine and levarterenol in concentrations of 1:50,000 to 1:800,000 and levo-nordefrin in concentrations of 1:20,000 to 1:320,000 prolonged the anesthetic effect of propoxycaine. This increase was proportional to the concentration of the vasoconstrictor (Fig. 2). The epinephrine and levo-nordefrin regression lines were nearly parallel, epinephrine being approximately twice as active as the latter. The slope of the levarterenol line was lower. At the highest concentration, the duration of anesthesia with the levarterenol solution was approximately half that of epinephrine, but the difference between the two drugs decreased with the concentration. Two ex-

* Nordefrin = Cobefrin(R) and levo-nordefrin = Neo-Cobefrin(R); available from Cook-Waite Labs., Inc.

TABLE 2

INTRADERMAL ANESTHESIA: DURATION TEST
(Propoxycaine HCl 0.25% + Vasoconstrictor)

Conc. of Vaso-constrictor	Epinephrine		Levarterenol		Levo-Nordefrin	
	Av. Duration (Min.)	Per Cent Increase	Av. Duration (Min.)	Per Cent Increase	Av. Duration (Min.)	Per Cent Increase
1: 20,000					374 (56)	568
1: 40,000					290 (62)	370
1: 50,000	390 (57)*	580	242 (78)	210		
1: 50,000	320†	360				
1: 80,000					218 (78)	180
1:100,000	270 (69)	290	198 (78)	154		
1:160,000					165 (81)	104
1:200,000	195 (66)	200	176 (78)	126		
1:320,000					98 (64)	55
1:400,000	114 (66)	73	117 (70)	67		
1:800,000	150 (68)	120	90 (64)	40		
1:800,000	94†	34	82 (†)	17		

* Figures in parentheses indicate duration of 0.25 per cent propoxycaine HCl (parallel test). The mean ± s.e. for the 15 tests run with this solution was 70 ± 2 minutes.

† These results were obtained with propoxycaine + epinephrine and propoxycaine + *l*-arterenol solutions run in parallel. The percentage increase in duration has been calculated by using the average propoxycaine control duration.

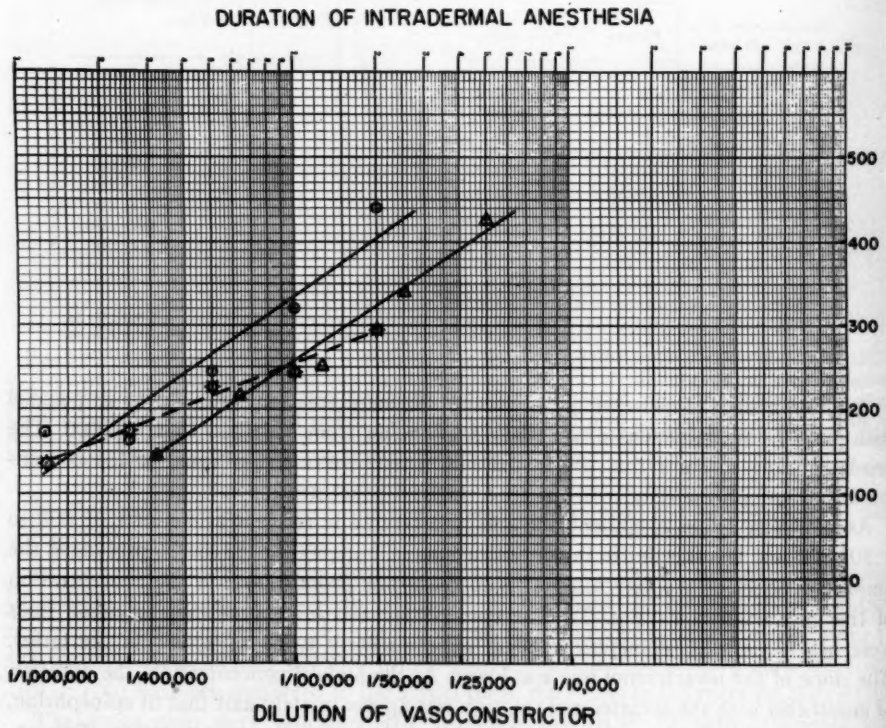

FIG. 2.—Regression lines obtained by plotting the mean duration of anesthesia in minutes (on the axis of ordinates) against the dilution of the vasoconstrictor in the solution (*abscissas*). Each solution contained 0. 25 per cent propoxycaine HCl in addition to the vasoconstrictor. Epinephrine: ⊙—⊙; l-arterenol; ☼—☼; l-nordefrin: △—△. The solution of 0.25 per cent propoxycaine HCl without a vasoconstrictor produces anesthesia of an average duration 70 ± 2min. This was the *grand mean* of 15 mean_1 values obtained with a total of 150 guinea pigs.

periments in which epinephrine and levarterenol solutions were tested in parallel (Table 3) tended to confirm the earlier results (Table 2). In this test, there was very little difference between the two drugs at the lower concentration (1:800,000). The duration of anesthesia with the epinephrine solution (1:50,000) was longer than that of levarterenol (1:25,000). The difference in the slope of the two curves could be explained by assuming either that epinephrine is more active* but is metabolized faster locally (only in very low concentrations) or that they are equally active but arterenol has a slightly faster rate of diffusion from the area of injection.

TABLE 3

INTRADERMAL ANESTHESIA: DURATION TEST

Experimental Solution	No. of Guinea Pigs	Conc. of Active Agents in Solution		Av. Duration of Anesthesia (Min.)
		Local Anesthetic	Vasoconstrictor	
1 A vs. B	10	Propoxycaine HCl 0.25%	Levarterenol 1:800,000	82
		Propoxycaine HCl 0.25%	Epinephrine 1:800,000	94
2 C vs. D	10	Propoxycaine HCl 0.25%	Levarterenol 1: 25,000	300
		Propoxycaine HCl 0.25%	Epinephrine 1: 50,000	320

TABLE 4

DURATION TEST

(Propoxycaine HCl + Epinephrine 1:100,000)

Conc. of Propoxycaine HCl (Per Cent)	No. of Guinea Pigs	Average Duration (Min.)					
		Experimental Solution		Control Solution*		Mean$_1$ Exp. Sol. ×100÷ Mean$_1$ Control Solution	P†
		Mean$_1$	Mean$_2$±s.e.	Mean$_1$	Mean$_2$±s.e.		
0.125	10	290	292±13.4	314	314±11.6	92	>0.1
0.5	10	363	351±15.2	315	321±13.6	116	>0.1
1.0	10	346	335±18.6	316	308±14.0	110	>0.1
2.0	10	350	347±13.3	290	288±10.0	120	<0.01

* Control solution = Propoxycaine HCl 0.25 per cent + epinephrine 1:100,000.

† P of difference between experimental and control mean$_2$ values.

In another series of experiments, a solution containing 0.25 per cent propoxycaine HCl and epinephrine 1:100,000 was tested in parallel with other solutions with the same concentration of epinephrine and graded concentrations of propoxycaine HCl. The results, which are presented in Table 4, show that the duration of anesthesia produced by the 0.5 per cent solution of propoxycaine was 25 per cent longer than that following the injection of the 0.125 per cent solution. However, no further increase

* In this case the same degree of vasoconstriction would be produced by a smaller concentration of epinephrine. Local inactivation of epinephrine and arterenol would decrease percentagewise as the concentration of the vasoconstrictor increases.

in duration was obtained by another fourfold increase in the concentration of the local anesthetic.

Procaine HCl solutions containing epinephrine 1:100,000 were tested as in the propoxycaine experiments, with the exception that the control solution was 0.5 per cent procaine HCl (plus 1:100,000 epinephrine). No significant prolongation was observed when the concentration of procaine HCl was increased from 0.25 per cent to 2.0 per cent. However, a 20–22 per cent decrease in duration occurred when the concentration was raised to 4.0 per cent.

Although the results obtained with propoxycaine and procaine were similar, the absolute duration of propoxycaine anesthesia was much longer than that of procaine. Obviously, it was not due to differences in potency, since solutions of 0.125 and 0.25 per cent have approximately the same anesthetic potency as procaine solutions of 1 and 2 per cent, respectively, propoxycaine being seven to eight times more active than procaine. Procaine may simply diffuse out of the wheal faster than propoxycaine, or it may increase the diffusion rate by antagonizing the vasoconstrictor effect of epinephrine. The experiments summarized in Table 6 show that the duration of propoxy-

TABLE 5

DURATION TEST

(Procaine HCl + Epinephrine 1:100,000)

Conc. of Procaine HCl (Per Cent)	No. of Guinea Pigs	Average Duration (Min.)					
		Experimental Solution		Control Solution*		Per Cent Control $Mean_1$ Exp. Sol. $\times 100 \div$ $Mean_1$ Control Solution	P†
		$Mean_1$	$Mean_2 \pm$ s.e.	$Mean_1$	$Mean_2 \pm$ s.e		
0.25	10	178	172± 4.2	166	183± 6.4	107	>0.1
1.0	10	225	231±10.7	229	229±10.4	100	>0.1
2.0	10	205	195± 8.3	187	204± 5.9	110	>0.1
4.0	10	168	196±19.6	174	167± 4.7	97	>0.1

* Control solution = Procaine HCl 0.5% + epinephrine 1:100,000.

† P of difference between experimental and control mean$_2$ values.

TABLE 6

DURATION TEST

Experiment	No. of Guinea Pigs	Solution	Concentration of Active Agents in Solution			Duration of Anesthesia (Min.)		
			Propoxycaine HCl	Procaine HCl	Epinephrine	$Mean_1$	$Mean_2 \pm$ s.e.	P
1	10	A	0.25	1.0	1:100,000	240	232 ±11.2	
		B	0.25	.....	1:100,000	284	286 ±12.0	<0.01
2	10	C	0.25	2.0	1:100,000	193	197 ± 4.1	
		D	0.25	.....	1:100,000	284	293 ± 9.8	<0.01
3	10	E	0.25	2.0	.....	65	62.5± 3.0	
		F	0.25	.....	.....	82	81.6± 4.1	<0.01

caine anesthesia is shortened by the addition of procaine to the solution. Therefore, there must be an increase in the diffusion rate of propoxycaine. This increase in the rate of diffusion of epinephrine and propoxycaine with reduction in the effect of epinephrine on duration (physiological antagonism) is probably the result of the vasodilator action of the procaine.

DISCUSSION

The experiments described here showed that, within a wide concentration range, increments in the duration of action of 0.25 per cent propoxycaine were produced by equal increments in the log concentration of the vasoconstrictor.

On the other hand, increments in the log concentration of propoxycaine and procaine produced only small changes in the duration of anesthesia. This is in agreement with the clinical results obtained by Winter and Tainter[5] with dental anesthetic solutions containing 2 and 3 per cent procaine HCl. However, Huldt[6] found a direct correlation between concentration of lidocaine and duration of dental anesthesia in solutions containing 1:40,000 epinephrine. These differences may result from differences in drugs tested and from the manner in which the presence of anesthesia was determined.

Our experiments showed that epinephrine reduced the minimal effective dose of propoxycaine. This effect of epinephrine on the action of local anesthetics has been observed by Braun[7] and Bülbring and Wajda,[2] among others, but not by Sollmann.[8]

Since the intradermal injection of epinephrine 1:100,000 produced only minimal anesthesia and the same solution plus 0.125 per cent propoxycaine HCl produced anesthesia lasting for 290 minutes (average), it could be asumed that at some point below this propoxycaine concentration, possibly very near the threshold anesthetic concentration 5 (Table 1), a certain degree of correlation might have existed between the concentration of the anesthetic and its duration of action.

The results shown in Table 2 strongly suggest that the duration of anesthetic action of mixed solutions of local anesthetics and vasoconstrictors depends mainly on the degree of blood flow in the injected area. The inherent ability of the compound to diffuse through the tissues may also play a part.[9]

SUMMARY

Intradermal anesthetic activity and duration of anesthesia were determined in guinea pigs by the method of Bülbring and Wajda.

The addition of graded concentrations of epinephrine (1:25,000 to 1:400,000) increased the local anesthetic activity of a solution of 0.25 per cent propoxycaine HCl, the greatest effect being obtained with the highest concentration.

Epinephrine, levarterenol, and levo-nordefrin increased the duration of propoxycaine anesthesia. Linear dose-effect curves were obtained by plotting the average duration in minutes against the log of the vasoconstrictor concentration in solutions of 0.25 per cent propoxycaine.

In solutions containing epinephrine 1:100,000, changing the concentration of procaine from 0.5 to 4.0 per cent or that of propoxycaine from 0.25 to 2.0 per cent did not greatly prolong the duration of anesthesia.

The duration of action of the procaine solutions was shorter than that of the propoxycaine solutions.

These results show that in mixtures of propoxycaine (or procaine) and vasoconstrictors, in concentrations similar to those found in commercial dental anesthetic solutions, the duration of local anesthesia is influenced much more by the concentration of the vasoconstrictor than by that of the local anesthetic.

The able technical assistance of Miss L. Jewell and Miss J. Radowicz is gratefully acknowledged.

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Combined Anticariogenic Effect of a Single Stannous Fluoride Solution and the Unsupervised Use of a Stannous Fluoride–containing Dentifrice II. Results at the End of Two Years

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It has previously been reported[1] that children six to fifteen years of age who used a stannous fluoride–containing dentifrice in an unsupervised manner had 32 per cent less tooth decay when the DMFT index was used to evaluate caries and 23 per cent less when the DMFS index was used than comparable controls using an identical dentrifice minus the stannous fluoride. When a single application of an 8 per cent stannous fluoride solution was used each 6 months in combination with the same stannous fluoride dentifrice, 58 per cent less caries was found when either the DMFT or the DMFS index was used. Such data agree with the in vitro studies[2] which suggest that the use of a stannous fluoride dentifrice prolongs the anticariogenic effect of topical stannous fluoride and that the use of a non-stannous fluoride–containing dentifrice reduces the duration of effectiveness by removing the protective tin phosphate layers of enamel.

Since the duration of effectiveness of topical therapy is of major importance, this study reports the results at the end of 2 years in a group of children receiving a single topical application of stannous fluoride and who, in addition, used the stannous fluoride dentifrice.

EXPERIMENTAL METHODS

The selection of the children, the balance of the three groups, and the conduct of the study have previously been reported in detail.[1] Briefly, Group 1, or the control group, received a single topical application of distilled water and used a non-fluoride–containing dentifrice, while children in Group 2 received a topical application of distilled water plus a commercially available stannous fluoride dentifrice.* The children in Group 3 received a single topical application of an 8 per cent solution of stannous fluoride at 6-month intervals by methods previously described[3] and, in addition, used a commercially available stannous fluoride dentifrice. None of the children received any instruction on the use, or the frequency of use, of the dentifrice but were told only to use it in accordance with their usual oral hygiene practices.

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* Crest brand dentifrice, the Procter and Gamble Co. The exact composition of this dentifrice is identical with that described by Jordon & Peterson (*J.A.D.A.*, **54**:589, 1957).

RESULTS

Tables 1 and 2 show the age and sex distribution of the subjects in this study. Table 1 shows the balance according to age and sex at the end of 18 months, and Table 2 a similar breakdown for the 24-month data.

The caries increments at the end of 18 and 24 months in the three groups according to the DMFT and the DMFS index are found in Table 3. For comparative purposes the 6- and 12-month data are also included in this table. At the end of 18 months the

TABLE 1

AGE AND SEX DISTRIBUTION OF SUBJECTS AT INITIAL EXAMINATION
(Only Subjects for Whom an 18-Month Examination Was Obtained)

AGE (YEARS)	CONTROL			SnF_2 DENTIFRICE			DENTIFRICE PLUS TOPICAL		
	Males	Females	Total	Males	Females	Total	Males	Females	Total
6	1	3	4	1	5	6	3	7	10
7	6	9	15	10	14	24	3	10	13
8	11	13	24	8	12	20	12	10	22
9	16	9	25	10	15	25	15	16	31
10	14	17	31	24	17	41	16	22	38
11	8	7	15	6	10	16	10	12	22
12	8	8	16	10	10	20	8	11	19
13	9	12	21	7	11	18	7	9	16
14	12	8	20	12	2	14	16	6	22
15	12	3	15	7	7	14	7	3	10
16	3	5	8	3	4	7	3	3	6
17	1	3	4	2	2	4	1	3	4
18	2	0	2	0	2	2	1	1	2
Total	103	97	200	100	111	211	102	113	215

TABLE 2

AGE AND SEX DISTRIBUTION OF SUBJECTS AT INITIAL EXAMINATION
(Only Subjects for Whom a 24-Month Examination Was Obtained)

AGE (YEARS)	CONTROL			SnF_2 DENTIFRICE			DENTIFRICE PLUS TOPICAL		
	Males	Females	Total	Males	Females	Total	Males	Females	Total
6	1	3	4	1	4	5	2	7	9
7	5	10	15	11	13	24	3	9	12
8	10	13	23	9	10	19	11	10	21
9	14	10	24	10	14	24	13	17	30
10	12	17	29	19	17	36	15	20	35
11	8	7	15	4	9	13	8	11	19
12	8	6	14	8	10	18	8	11	19
13	9	13	22	6	9	15	6	9	15
14	11	7	18	11	2	13	13	6	19
15	13	3	16	5	6	11	7	3	10
16	3	5	8	2	3	5	3	3	6
17	1	2	3	2	2	4	1	1	2
18	2	0	2	0	2	2	1	1	2
Total	97	96	193	88	101	189	91	108	199

control group had an increment of 2.04 in terms of the DMFT index and 4.72 new DMFS surfaces. The group using the stannous fluoride dentifrice who, in addition, received a topical application of water had 1.30 new DMF teeth and 3.43 new DMF surfaces, a reduction of 36 and 27 per cent, respectively, in terms of the DMFT or DMFS index when compared with the control group. The caries increment in the

TABLE 3

COMPARISON OF CARIES INCREMENTS OF THREE GROUPS IN TERMS OF DMFT AND DMFS INDEXES AT EACH OF FOUR EXAMINATION PERIODS

GROUP*	NO. OF SUBJECTS	DMFT INCREMENT	PER CENT REDUCTION	P	DMFS INCREMENT	PER CENT REDUCTION	P	REVERSALS IN DIAGNOSIS
	6-Month Data							
1.....	230	0.86	.....	.....	1.97	.....	.....	0.20
2.....	228	0.53	38	0.0085	1.53	22	0.058	0.30
3.....	233	0.34	61	0.0006	0.69	65	0.00006	0.46
	12-Month Data							
1.....	220	1.38	.....	.....	3.15	.....	.....	0.15
2.....	221	0.94	32	0.0048	2.44	23	0.025	0.35
3.....	230	0.58	58	0.0001	1.31	58	0.00001	0.49
	18-Month Data							
1.....	200	2.04	.....	.....	4.72	.....	.....	0.22
2.....	211	1.30	36	0.0006	3.43	27	0.004	0.33
3.....	215	0.84	59	0.0001	2.08	56	0.0001	0.53
	24-Month Data							
1.....	193	2.49	.....	.....	5.91	.....	.....	0.18
2.....	189	1.66	33	0.0001	4.41	25	0.001	0.23
3.....	199	1.04	58	0.00001	2.64	55	0.00001	0.50

* Group 1 = water, topical, plus control dentrifice; Group 2 = water, topical, plus SnF_2 dentrifice; and Group 3 = SnF_2, topical, plus SnF_2 dentrifice.

group receiving both the topical stannous fluoride treatment and the stannous fluoride–containing dentifrice was 0.84 new DMF teeth and 2.08 new DMF surfaces, or a caries reduction of 59 and 56 per cent, respectively, when compared with the controls. The reductions in both those groups and for both indexes are statistically significant, as shown by their low probabilities.

DISCUSSION

It is of considerable interest to compare the effectiveness of these treatments as a function of time. One will note that the reductions obtained at the end of 2 years are,

for all practical purposes, identical with the data at each of the three preceding examination periods. There was no decrease in effectiveness of either the results obtained with the stannous fluoride dentifrice or in the group using a combination of topical stannous fluoride treatment and the daily use of a stannous fluoride dentifrice. These results suggest, therefore, that the reapplication of an 8 per cent solution of stannous fluoride every 6 months and the daily use of a stannous fluoride dentifrice produce a significant reduction in dental caries in children—an effect which does not decrease with time. Of course, these conclusions apply to the period of only 2 years, and extrapolation beyond this period is not warranted until additional data are available.

SUMMARY

The routine use of a commercially available stannous fluoride–containing dentifrice in those subjects who receive a single application of an 8 per cent solution of stannous fluoride each 6 months was associated with a significant reduction in dental caries. This anticariogenic effect did not decrease in magnitude during a 2-year study period.

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Dental Eruption, Stature, and Weight of Hiroshima Children

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In the course of a broad study on the health of school children conducted in Hiroshima between October, 1958, and May, 1959, an increase in stature, weight, and dental eruption, as compared with similar data collected in 1951, was noted. The purpose of this paper is to point out the magnitude of this difference and to discuss, in a preliminary fashion, some of the factors which may have contributed to it.

TABLE 1

SECULAR CHANGES IN STATURE—A SUMMARY OF SOME PREVIOUS STUDIES

Source	Age (Years)	Period	Total Increase (Cm.)	Yearly Increase (Cm.)
Sweden:				
Abramson & Ernest	10.5–20.5	1883–1950	14.3	0.21
England:				
Clements	5–13	1910–47	6.25–8.75	0.24
Japan:				
Matsuyama, quoted in Markowitz	13–18	1900–1940	4–5	0.13
U.S.:				
Meredith	10	1892–1952	8	0.13
France:				
Tremolières *et al.*, quoted in Krogman	20	1895–1900 to 1946–48	5	0.10
Japan:				
Hiroshima Prefecture Statistical Office	Approx. 10	1950–58	3.35	0.42

Secular changes in stature and weight have been observed numerous times.[1–5] Table 1 presents a summary of a few previous studies. Some of these studies, as well as others, have noted an effect of socio-economic status on growth.[1, 2, 5–7]

No previous work dealing specifically with secular changes in tooth eruption has

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come to the attention of the authors. Several studies, however, have been concerned with the effect of socio-economic status on the process of dental eruption. Clements[8, 9] reported a trend toward earlier eruption in children of superior socio-economic classes. He also suggested a tendency toward earlier tooth eruption in 1948, as compared with 1925. Adler[10] stated that "well-to-do" children showed advanced dental eruption and that urbanization was an accelerating factor. On the other hand, Hellman[11] concluded that children from wealthy families were slower in tooth eruption than children from poor families but were more accelerated in other aspects of growth.

It is not the purpose of this paper to quantify or even define any of the factors responsible for the observed growth changes. Discussion in this regard is, for the most part, speculative and is included to indicate some of the areas in which future work might be enlightening.

EXPERIMENTAL METHODS

The 1959 data were obtained in the course of the Child Health Survey conducted at the Atomic Bomb Casualty Commission* and included only those children seen between October and May, 1959. The primary purpose of the Child Health Survey was to study the effects of inbreeding. Only children from the control group of the Child Health Survey, who were examined within 6 weeks of their birthdays, were included in the analyses presented below. The 6-week restriction was placed so as to approximate as closely as possible the age of the group examined in 1951. All children in the 1958–59 study were born in Hiroshima of parents who had received no significant radiation exposure. Children with known disease or defects which might affect their growth were excluded, as was done in 1951.

The control group of the Child Health Survey represents a systematic sample of approximately 8 per cent of the children whose parents received either no radiation or very light exposure and were included in the previous genetic program of the ABCC.[12] In this genetic study, approximately 93 per cent of all live-born children in Hiroshima between 1948 and 1954 were examined.

Each child seen by the Child Health Survey received physical, anthropometric, and dental examinations. Stature and weight were obtained by using a standard physician's clinic scale and standing-height anthropometer. The criterion for an erupted tooth was identical with that used in 1951; i.e., if any portion of the crown was visible, the tooth was considered erupted. Social and medical histories were obtained prior to examination.

The comparison group was taken from the controls of the 1951 pediatric program of the ABCC (children conceived prior to the explosion of the atomic bomb). Complete descriptions of this program can be found in ABCC reports.[13] Anthropometric data were taken from the work of Reynolds[14] and dental data from Sutow, Terasaki, and Ohwada.[15] It was necessary to use original data on file at the ABCC to obtain values for the variances of the mean numbers of erupted teeth published by Sutow.

The 1951 and 1959 samples differ, in that about 20 per cent of the early group were born outside Japan and a total of about 30 per cent lived for an extended period outside Hiroshima city. It was assumed that the difference in birthplace and early residence would not invalidate comparisons of the two groups.

* Hereafter abbreviated "ABCC."

RESULTS

Table 2 shows a comparison of the anthropometric and dental findings for the two groups. In all cases the children were taller, heavier, and dentally more advanced in 1959. Levels of significance of the differences in number of erupted teeth for the eight- and ten-year-olds were determined by the *t*-test. In all cases the differences were statistically significant.

No attempt was made to determine significance for the six-year-olds because no figures were available from which to compute the variance for the 1951 group. The differences in the means for the six-year-olds, if tested, might prove not to be signif-

TABLE 2

NO. OF PERMANENT TEETH, STATURE, AND WEIGHT OF HIROSHIMA CHILDREN 6, 8, AND 10 YEARS OF AGE; 1959 VERSUS 1951

	Boys							Girls						
	1951			1959			*P*	1951			1959			*P*
	N	$\bar{X}$	S.D.	*N*	$\bar{X}$	S.D.		*N*	$\bar{X}$	S.D.	*N*	$\bar{X}$	S.D.	
No. Permanent teeth:														
6 yr.	89	1.59	.....	26	2.12	2.55	.....	81	2.31	.....	21	3.52	2.42	.....
8	107	8.60	2.40	32	9.78	2.46	.01	106	9.36	2.25	23	10.52	1.83	.025
10	99	14.55	3.68	39	16.34	3.59	.005	101	15.55	3.80	39	19.00	4.12	.005
Stature (cm.):														
6 yr.	83	106.58	4.85	26	109.01	4.62	.....	77	105.65	4.44	21	108.91	3.37	.....
8	89	117.54	4.08	32	119.59	4.58	.....	101	115.94	4.42	23	118.16	3.87	.....
10	89	125.90	5.11	39	128.97	4.41	.....	87	126.08	5.91	39	128.49	4.19	.....
Weight (kg.):														
6 yr.	83	17.24	1.80	26	18.73	1.65	.....	77	16.68	1.66	21	18.35	2.06	.....
8	89	20.86	1.90	32	22.02	2.22	.....	101	20.31	2.18	23	21.38	2.40	.....
10	89	24.97	2.38	39	25.97	2.31	.....	87	24.47	2.67	39	25.87	2.60	.....

icant at the 5 per cent level but are, nevertheless, in the same direction as the eight- and ten-year-olds and do not detract from the hypothesis that teeth were erupting earlier in 1959.

Figures published by the Hiroshima Prefecture Statistics Department for the same period confirm these findings in regard to height and weight.[16] Their height-weight tables from 1950 to 1958 for Hiroshima Prefecture school children from six to eleven years of age showed an average increase of about 2.65 cm. in stature and 0.7 kg. in weight. The six-year-olds showed a somewhat smaller increase, whereas the ten-year-olds gained a greater amount. These data also suggest that the greatest increase occurred in the early years. No comparable figures on tooth eruption were available for comparison.

DISCUSSION

One factor which has been mentioned as a cause of secular changes in growth is the breaking-up of population isolates and the subsequent decrease in inbreeding.[1] Other

factors are improved nutrition, sanitation, and medical care. The latter factors and many others can be considered as effects of improved social and economic conditions.

The situation in Hiroshima during the period in question was one of continual and rapid economic improvement; therefore, a pronounced effect on growth might be expected. The average monthly income per household rose from approximately 14,000 yen ($39.00) in 1950 to almost 40,000 yen ($111.00)* in 1958.[17] The portion of the family income spent for food fell from about 43 to 28 per cent.[17] The frequency of intestinal parasites in this age group is, at present, about 20 per cent, compared with 40 per cent in 1953.[16] There is little doubt that, in respect to physical environment, the children of Hiroshima are today at a great advantage over children of a few years ago.

The effect of caries in the primary dentition on the eruption of the permanent teeth is a problem which has concerned several authors.[18–20] Does premature loss of deciduous teeth through caries result in early eruption of permanent teeth in the unoccupied space? If it does, the acceleration in tooth eruption observed in this study might be partially accounted for on this basis. Figures published by the Ministry of Education for all Japan for six- to twelve-year-olds show dental caries prevalence to be almost twice as great in 1957 as in 1949.[21] The percentage of boys with dental caries experience was 43.39 in 1949, compared with 76.70 in 1957. For girls the figures were 41.75 versus 76.70. Data collected in the course of the Child Health Survey place the prevalence in excess of 90 per cent for both sexes in 1959.

Do children have earlier eruption and hence more teeth at given age levels because of more caries, or more caries because of the greater number of teeth in these age classes? In a study of New Zealand children, Leslie[18] found a significant increase in the number of erupted premolars in those individuals who had experienced extraction of deciduous molars, compared with a group, similar in age and sex, who had had no extractions. Premature loss of deciduous teeth was a common finding in Hiroshima. Although there seems to be some justification for speculating that part of the observed acceleration in tooth eruption resulted from increased dental caries, with an accompanying increase in premature loss of deciduous teeth, how much of it was due to increased dental caries and how much to improved socioeconomic conditions or other, unrecognized, factors cannot be determined from the present data. A well-controlled serial study would be helpful in evaluating these influences.

Many additional questions regarding the relative importance of various factors in tooth eruption and other growth processes and the interrelationships of these factors remain unanswered. The effects of inbreeding, chronic illnesses, and the relationship of tooth eruption to other variables, such as stature, weight, and skeletal maturation, are some of the issues which warrant future study. Data which will be useful in studying many of these problems are now being collected on approximately 7,000 children in Hiroshima and Nagasaki.

SUMMARY

Data on dental eruption, stature, and weight of Hiroshima children in 1951 were compared with similar data collected in 1958 and 1959. The resultant increases in

* One dollar buys more calorie-vitamin needs in Japanese foods than the same amount would buy of American-European equivalents.

these measurements were noted, and some of the possible factors responsible were discussed. Several specific areas in which future work on tooth eruption might be promising were pointed out.

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Preliminary Report on the Mineralization of Human Dentin

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In spite of earlier extensive studies with the electron microscope, very little information has been gained concerning the inorganic phase of human dentin. A main reason for this lack of data is thought to be difficulty in specimen preparation. While more or less clear outlines of the crystalline structures in enamel have been brought out by replica technics,[1] the methods have not been applied with equal success to the mineral components in dentin. Furthermore, it has not been possible to make useful thin sections of fully calcified dentin with the glass or steel microtome knives which have been available. Some of the difficulties have been circumvented through the use of softer developing dentin,[2, 3] but the full course of mineralization has not yet been thoroughly explored.

One of the important recent advances in ultra-sectioning technics has been the introduction of the diamond knife.[4-7] Thin sections of undemineralized, fully calcified tissue, even enamel, can now be cut. This is making possible more extensive and systematic studies of mineralization. The present paper is a preliminary report on the mineral component of developing and mature human dentin.

MATERIALS AND METHODS

The majority of the specimens were partly mineralized deciduous tooth germs dissected from formalin-fixed human fetuses. The ages of the fetuses ranged from 3 to 4½ months. Some dentin from formalin-fixed, non-carious, fully calcified permanent molars was also used. Additional samples were obtained from unfixed, non-carious, fully calcified third molars rendered anorganic by ethylenediamine treatment.†

The samples were dehydrated and imbedded in an 8:2 mixture of butyl and methyl methacrylates, following the usual procedures. A microtome‡ equipped with a diamond knife was used for sectioning. Sections were picked up on specimen screens previously covered with carbon substrates and were subjected to electron microscopy and diffraction without removal of the imbedding medium. In some instances the sections were decalcified with 0.1 N HCl for a few seconds on the specimen screens.

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† Some of the anorganic dentin was provided by Dr. F. L. Losee, National Naval Medical Center, Bethesda, Maryland (see Refs. 8–10).

‡ Porter-Blum.

RESULTS

Developing dentin.—In the organic matrix near the basal end of the developing tooth germ, various small areas can be seen which are infiltrated with dot- or needle-shaped particles (Fig. 1, *A*). It is interesting to note the occasional accumulation of particles at the periphery of the odontoblastic processes (Fig. 1, *B*), which thus become encircled by a narrow, dense layer.

The areas infiltrated by the dense particles increase in size and fuse (Fig. 1, *C*). Around the odontoblastic processes the layer is gradually widened, and its density increases because of the additional accumulation of particles (Fig. 1, *D*). The light round spots in Figure 1, *B* and *D*, are probably cross-cut matrix fibrils which have been outlined as the result of deposition of particles in the interfibrillar areas. Inside these cross-cut fibrils and scattered in various areas a few hazy granules can be seen.

The particles mentioned above are so small that accurate description of their dimensions is difficult. The needles, however, seem to reach 200–800 A in length, and the granules are roughly 100 A or less in diameter. When areas showing these particles are subjected to diffraction, the presence of apatite can be detected. Further, the particles disappear when the section is decalcified on the specimen screen, and diffraction patterns can no longer be obtained. It is thus evident that the particulate matter contains apatite, but it is not yet certain whether or not individual particles are single crystals.

Occlusal to the area from which the previous findings were made, dentinogenesis had progressed to a point where distinct layers of calcified dentin and predentin could be distinguished. The junction between the two is quite abrupt (Fig. 2, *A*). Further deposition of mineral seems to take place appositionally from this dentino-predentinal junction.

Under higher magnification it can be seen that the mineral particles at the junction are identical with those seen in Figure 1, *A* (Fig. 2, *B*). There are more or less definite indications that the needles accumulate into dense aggregates through side-by-side alignment. Between these aggregates, granular particles similar to those shown in Figure 1, *D*, can be seen.

One of the conspicuous findings in the dentin is the regular deposition of crystals on the matrix fibrils. Consequently, the fibrils become characterized by alternately arranged dark and light cross-bands (Fig. 2, *C*). Closer observation gives an impression that the dark bands are composed of rows of particles in the shape of platelets. The fibrils seem to be mineralized to a lesser degree than the interfibrillar regions.

In the dentin the areas immediately surrounding the odontoblastic processes (so-called peritubular areas) are easily distinguished from the rest of the intertubular substance by their more compact and homogeneous texture (Fig. 2, *D*).

Mature dentin.—The findings obtained from untreated mature dentin are quite similar in many respects to those from the developing dentin. There are, however, some significant differences between the two.

The peritubular area, especially, shows an extremely dense structure which is almost impossible to resolve (Fig. 3, *A*). The boundary between the peritubular and intertubular areas becomes very sharp, and the former appears as a ring-shaped structure. The bands on the matrix fibrils are seen in the intertubular area (Fig. 3, *A* and

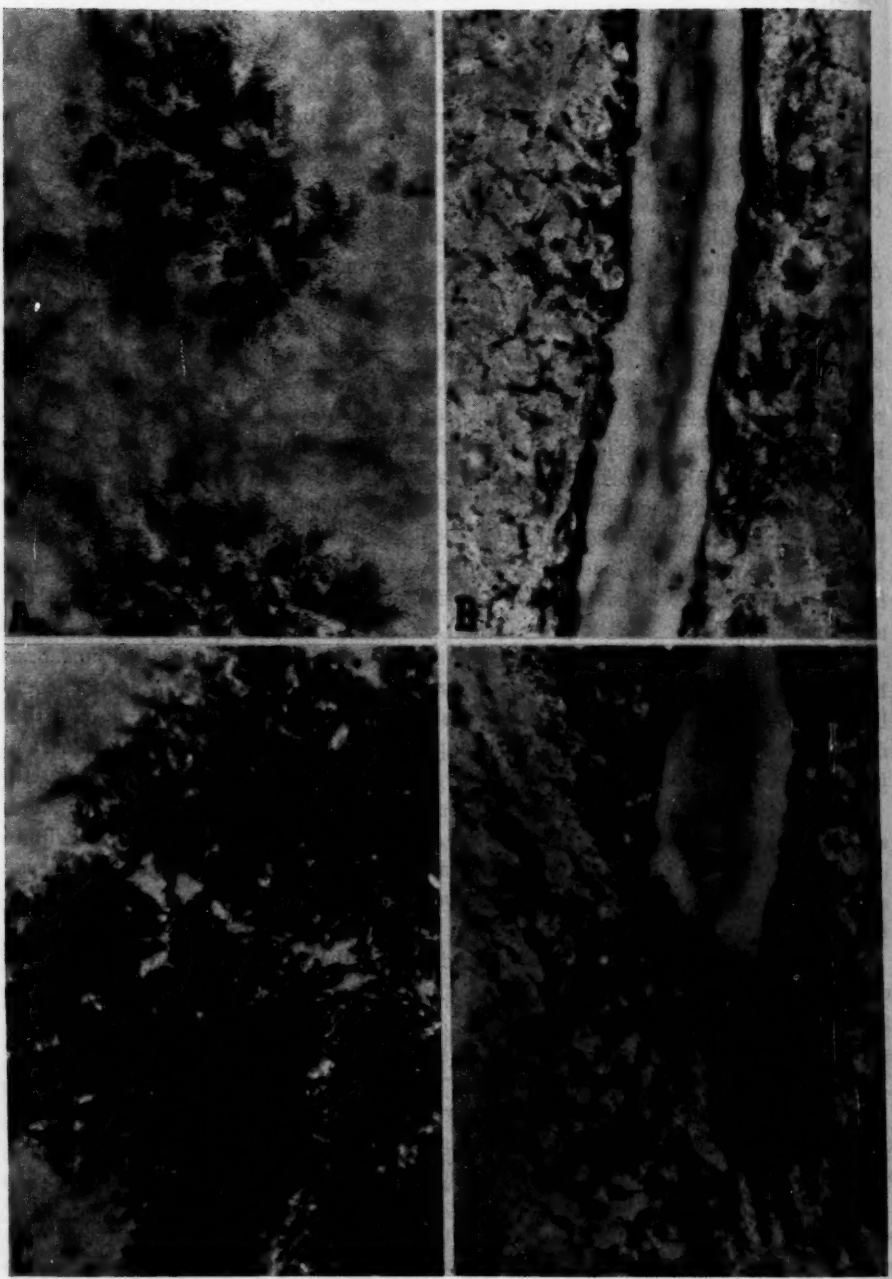

FIG. 1.—Early stages of dentin mineralization near basal end of deciduous tooth germ. *A:* Cluster of needle- and dot-shaped particles. (Mag. ×45,000.) *B:* Accumulation of particles at periphery of tubule. (Mag. ×22,000.) *C:* Confluence of mineralized areas. (Mag. ×22,000.) *D:* Increase in peritubular mineralization. (Mag. ×45,000.)

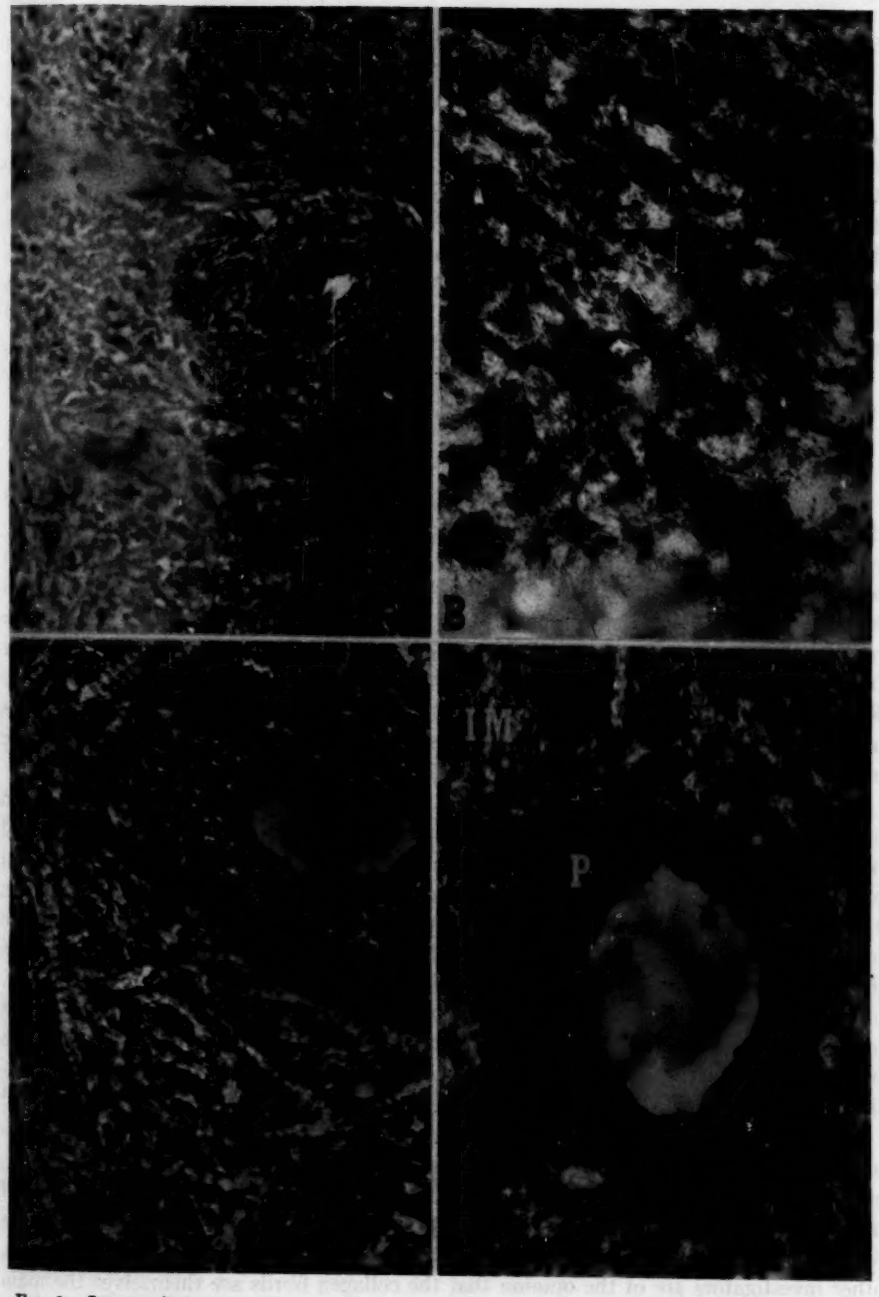

FIG. 2.—Intermediate stage of mineralization in mid-region of developing deciduous tooth crown. *A:* Border between predentin (*left*) and dentin (*right*). (Mag. ×12,000.) *B:* Higher magnification of dentin close to predentin border. (Mag. ×45,000.) *C:* Mineral deposition on collagen fibrils. (Mag. ×22,000.) *D:* Persistence of density difference between peritubular (*P*) and intertubular (*IM*) areas. (Mag. ×45,000.)

B), but in many regions they become obscured because of additional crystal deposition (Fig. 3, *C*).

In anorganic dentin the intertubular areas are filled with dense spindle-shaped or tabular objects (Fig. 4, *A*) which are considerably larger than the crystalline particles observed in developing dentin. Thus the spindle-shaped objects range from 330 to 2,200 A in length, and from 50 to 220 A in width, while the tabular objects vary in diameter from 110 to 300 A (Fig. 4, *B*). Although the spindle-shaped objects are very dense, there are indications that they may be composed of much smaller elongate units with fine cross-striations (Fig. 4 *C*). The impression is given by comparing Figure 4, *B*, with Figure 3, *B*, *C*, and *D*, that the above-mentioned tabular objects are related mainly to the matrix fibrils, and the spindle-shaped objects to the interfibrillar area.

The peritubular area appears as a highly mineralized region composed of closely packed, fine, round particles (Fig. 4, *D*), varying from 80 to 150 A in diameter.

DISCUSSION

During the present studies, great variation was noticed in the shapes and sizes of the mineral particles in dentin. The fine particles found in developing dentin may very well be single crystals, since their dimensions are the same as those obtained previously for crystals in dentin, bone, and cartilage by electron microscopy[11-17] and X-ray diffraction.[18, 19] The larger mineral particles observed in mature dentin may result either from aggregation of the smaller crystals seen in developing dentin or from an increase in crystal size with advancing age, as pointed out for bone by Robinson and Watson.[13] Furthermore, it is possible that the sizes and shapes of the inorganic components are related to the submicroscopic configuration of the organic matrix, as suggested by Nylen, Scott, and Mosley.[20]

Besides the size of the mineral particles, other differences between developing and mature dentin were noticed. In the former the border between the highly mineralized peritubular area and the less mineralized intertubular area is usually transitional. In mature dentin, however, the border is quite abrupt, and the peritubular area appears as a well-defined ring-shaped structure of much higher density. The periodic structure on the matrix fibrils which can be seen in developing dentin is obscured in many places in mature dentin, probably because of additional mineral deposition.

On the basis of these findings, it could be said that calcification of dentin, at least in the early stages, proceeds through progressive deposition of mineral in the dentin proper, as well as through apposition at the dentino-predentinal junction. Such a continuous mineralization has also been suggested in recent radioautographic studies.[21]

Opinions concerning the actual portion of the calcified tissues occupied by the inorganic components have been somewhat controversial, and the matter is still unsettled, even after electron-microscopic observations. Some researchers claim that the mineral in bone is localized primarily in the interfibrillar area and that the matrix fibrils are retained in an almost fully or completely unmineralized state.[22, 23] Contrary to this, other investigators are of the opinion that the collagen fibrils are themselves the main bearers of the bone crystals.[11-15, 24, 25] Watson and Avery concluded that the initial mineralization in dentin seems to take place within the matrix fibrils and later extends to fill the interfibrillar region.[2] It is evident from the present work that mineralization

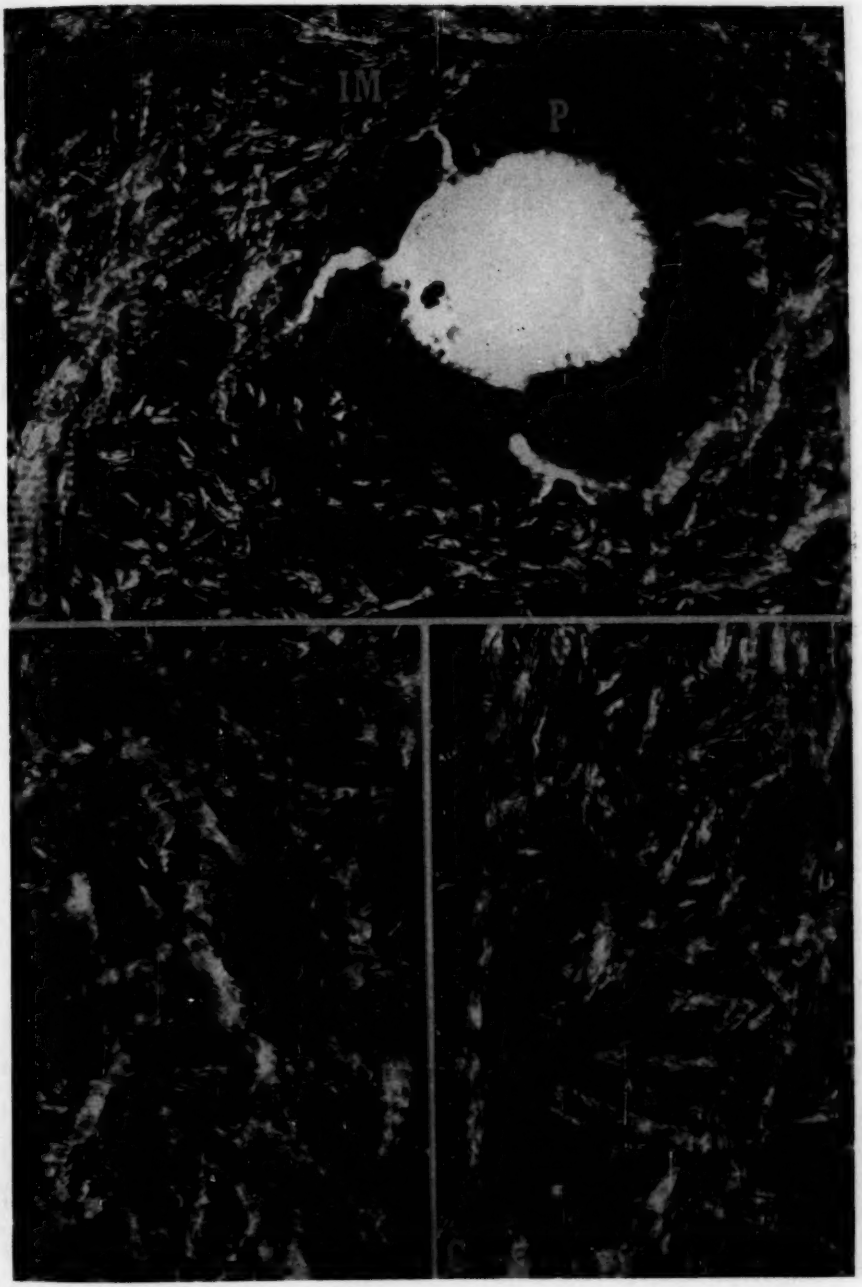

FIG. 3.—Untreated, fully mineralized dentin from erupted, permanent tooth. *A:* Peritubular area (*P*) appearing extremely dense and sharply demarcated from surrounding intertubular matrix (*IM*). (Mag. ×24,000.) *B:* Enhanced cross-banding of matrix fibrils due to initial mineral deposition. (Mag. ×38,000.) *C:* Partial obliteration of striation pattern following additional mineral deposition. (Mag. ×38,000).

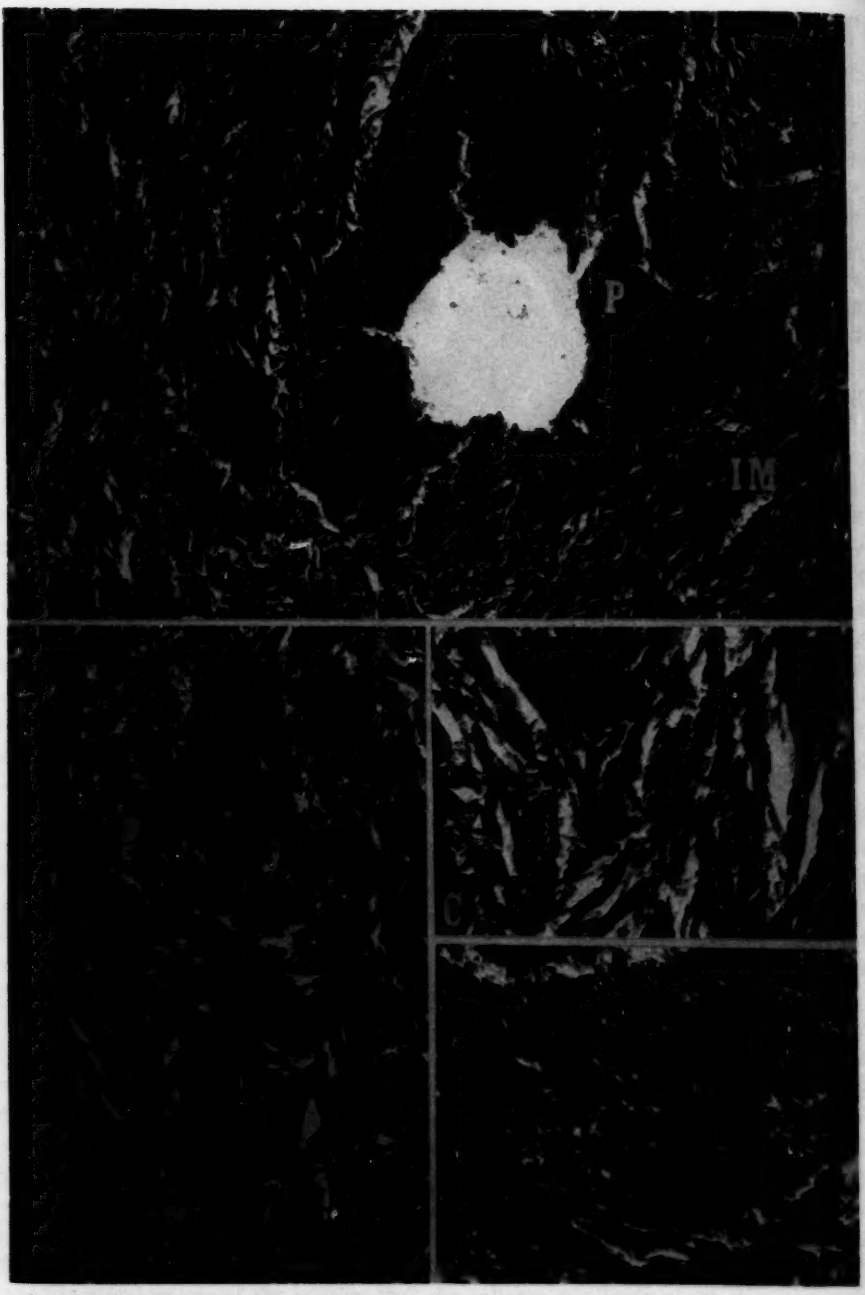

FIG. 4.—Fully mineralized dentin from which the organic components were removed through ethylene diamine treatment. *A:* Difference in amount of mineral present in peritubular (*P*) and intertubular (*IM*) areas. (Mag. ×30,000.) *B:* Spindle-shaped and tabular objects in intertubular region. (Mag. ×45,000.) *C:* Higher magnification of area similar to *B*. Mineral objects appear to be composed of smaller units. (Mag. ×100,000.) *D:* Fine particles observed in peritubular area at high magnification. (Mag. ×100,000.)

can occur not only in the interfibrillar area but also within the matrix fibril itself. The location in which crystals first appear, however, was not determined.

As has been pointed out,[11-16, 20, 24, 25] the fact that the minerals are deposited regularly on the collagen fibrils in a striated pattern would suggest that they are closely related to the original periodic structure in the matrix fibrils.

The existence and structure of the highly mineralized band (peritubular matrix) around the dentinal tubules have been described in previous replica studies.[26] Although information on its development is still scanty, the present studies seem to indicate that this structure has its origin during the early stages of dentin mineralization. Further studies have to be done to determine whether or not the mineralization of the peritubular matrix is preceded by changes in the organic matrix, as reported by Bradford.[27]

SUMMARY

Undecalcified ultra-thin sections of the developing and fully calcified human dentin were observed under the electron microscope. Preliminary findings on the mineralization processes in the developing dentin were presented. Some differences between the mineralization in immature and mature dentin were also suggested.

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Electron Microscopy of the Structure around the Dentinal Tubule

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It is well known that, when ground sections of dentin are examined under the optical microscope, a ring-shaped transparent zone can be seen around the odontoblastic processes. Although they differed in their opinions concerning its structure and composition, Neumann, Walkhoff, and Römer[1] interpreted this ring-shaped area as a special entity. On the contrary, Fleishman[1] claimed that the zone was a void produced through shrinkage of the odontoblastic process, and Hanazawa[2] stated that it was an optical illusion. In fact, no one is yet sure whether any of the contradictory opinions is correct, but for many years it has been generally accepted that there is no special structure around the odontoblastic process.

In recent years, however, the idea of such a special structure around the odontoblastic process has been revived. An important stimulus has been the new evidence brought out in studies with the electron microscope. In 1950, Menke[3] examined human dentin by the replica method and noticed a disk-shaped structure around the odontoblastic process which he considered to be softer than the remaining matrix. A similar structure was reported independently by Takuma and Tsuchikura,[4] who, in contrast with Menke, suggested that it is even more highly calcified than the rest of the dentin. After that, findings in agreement with the latter suggestion have been reported by other electron microscopists.[5–10a]

Paralleling these observations, important evidence of the existence of a special structure around the odontoblastic process has also been gained through optical microscopy[11–15] and microradiography.[16–19]

This special structure is referred to in various terms by different investigators—for example, "peritubular matrix" (Takuma and Tsuchikura[4]), "translucent area" (Bradford[11]), "hypercalcified ground substance" (Rouiller *et al.*[6]), "calcified sheath" (Shroff *et al.*[8]), "peritubular dentin" (Fearnhead[10]), "peritubular translucent zone" (Blake[18]), "tubulo-fibrillar complex" (Frank[20]). It is still too early to evaluate the terminology, especially since the developmental aspects of this structure are unsettled. In the present paper, therefore, the term "peritubular matrix" will be used tentatively, according to the previous paper.[4] It should be remembered, however, that the peritubular matrix is located within the confines of the dentinal tubule as established earlier by optical microscopy. The term "dentinal tubule" used in this paper implies the lumen surrounded by the peritubular matrix; "intertubular matrix" refers to the regions external to the peritubular zone.

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The present report is an electron-microscopic survey of this structure as revealed by special preparatory technics and reproduced by improved replica methods.

MATERIALS AND METHODS

Eighty-five formalin-fixed non-carious teeth extracted from ten- to sixty-year-old individuals were used. The sample was composed as follows:

	Incisors	Premolars	Molars
Erupted permanent teeth	9	28	34
Unerupted permanent teeth	2	.....	4
Deciduous teeth	3	.....	5

Acetylcellulose-carbon two-stage replicas were made from the surfaces of mechanically cleaved fragments or ground sections of crown dentin. Some of the surfaces of the ground sections were replicated after polishing alone; others were, in addition, subjected to preliminary treatment with acids or organic solvents.

The fine polishing of the ground sections was carried out on a rotating felt lap saturated with a slurry of fine gamma alumina. Acid etching was done with 0.01–0.3 N hydrochloric acid, in which the polished ground sections were immersed for 5–15 seconds. For the superficial removal of organic matter, the sections were boiled for 30–60 minutes in ethylenediamine.[21–23] Some dentin was available which had been rendered completely "anorganic" through long extraction with ethylenediamine. This material was mechanically cleaved, and replicas were made of the fracture surfaces.

Various combinations of treatments were also tried. For example, in some cases, one half of the polished dentin surface was treated with the acid, the other half was left unetched. In other cases the two halves of the dentin surface were exposed to successively stronger acid treatment. Observation of the junction areas permitted a comparison of the structures brought out by the different treatments.

The final carbon replicas were shadowed with tungsten oxide or palladium. The films were thin enough that the detail was equally well brought out when either the true replica surface or the reverse face was shadowed. In the former instance the structures reproduced were in their actual orientation, while in the latter they were obviously inverted. Some specimens were subjected to limited area electron diffraction to determine whether the material often picked up from the dentin surfaces and retained *in situ* on the replicas was mineral.

RESULTS

Figure 1 shows the acid-etched surfaces of dentin which were obtained from erupted deciduous (*A*), unerupted permanent (*B*), young erupted permanent (*C*), and old erupted permanent (*D*) teeth. The odontoblastic processes are seen as the most elevated projections, and the intertubular matrix appears fibrillar and granular. Between these two structures there are slightly elevated ring-shaped areas with a finely granular texture. These are the regions that are called "peritubular matrix" in the present paper. As illustrated, the peritubular matrix can be observed, irrespective of type, age, and eruptive status of the tooth.

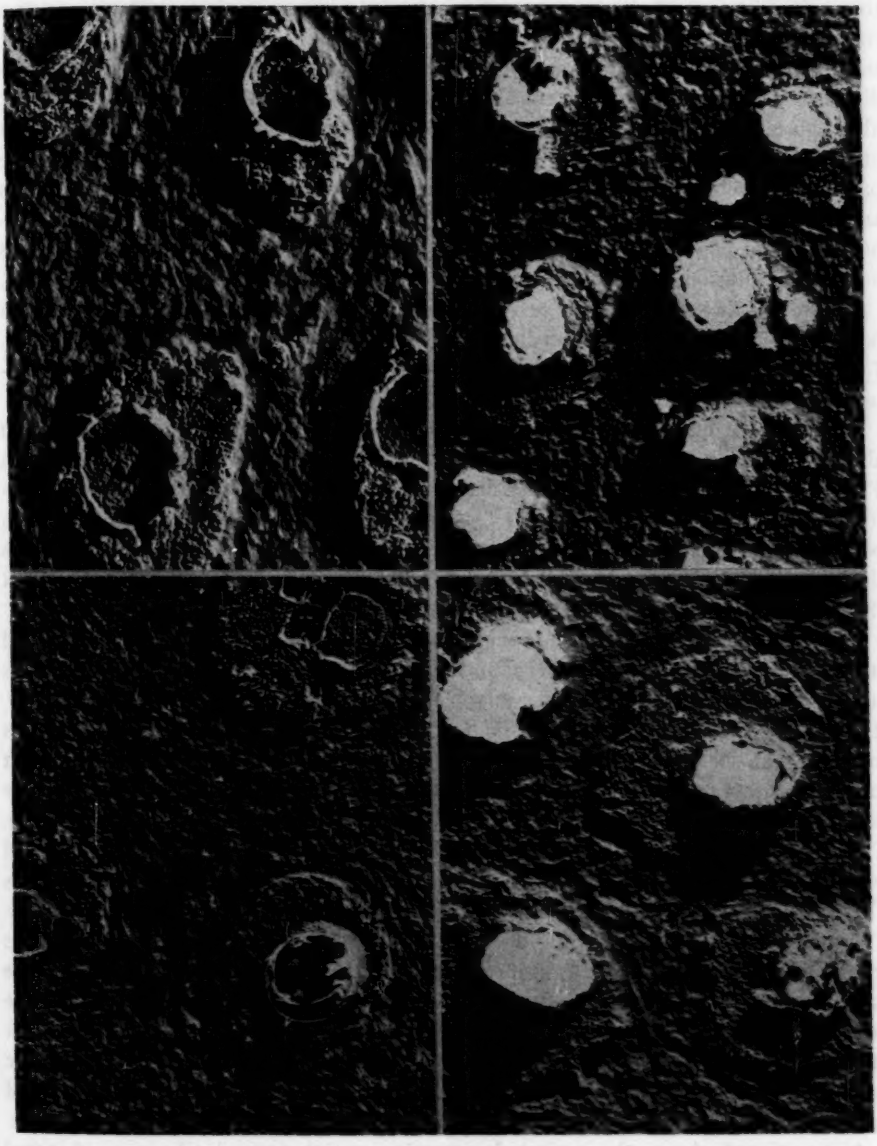

FIG. 1.—Peritubular matrix revealed on acid-etched cross-cut dentin surfaces from erupted deciduous (*A*), unerupted permanent (*B*), young erupted permanent (*C*), and old erupted permanent (*D*) teeth. (Etched 5 sec. with N/100 HCl; mag. ×9,500.)

The polished surface of dentin in which the tubules are sectioned transversely is shown in Figure 2, *A*. The peritubular matrix is seen as an extremely smooth area compared with the intertubular matrix. Figure 2, *B*, was obtained from a cleavage surface of dentin which had previously been completely de-organized with ethylenediamine. The dentinal tubules are also seen in cross-section. The peritubular matrix, although not so clearly seen as in Figure 2, *A*, can still be distinguished from the rest of the matrix. It is difficult to decide whether the texture shown here reflects the pattern of the inorganic component which remained or whether it actually represents an impression of the organic constituents left on the mineral phase after their dissolution. These observations do demonstrate, however, that the peritubular matrix is not a mere illusion but is a real structure around the lumen of the dentinal tubule.

Figure 2, *C*, was taken from the surface of a polished dentin section which had been boiled for 60 minutes in ethylenediamine. Compared with the untreated surface (Fig. 2, *A*), the intertubular matrix has become remarkably rough in texture. The peritubular matrix, however, still possesses its original smoothness.

A structural difference between the peritubular and the intertubular matrices can also be found on the surface of untreated, mechanically cleaved dentin, as shown in Figure 2, *D*. Again, the peritubular matrix has a much finer texture than the intertubular matrix. At the center of this picture, a longitudinally cut dentinal tubule can be seen, with tangled fibrils evident on its wall. Another example of these fibrils is shown at higher magnification in the upper left half of Figure 3, *A*. It is evident that they are collagen fibrils with regular 640 A striations.

The peritubular matrix can be brought out more clearly through acid treatment and stands up at a slightly higher level than the intertubular matrix in "negative" replicas such as those illustrated in Figures 1, *A–D* and 3, *B*. In "positive" replicas, however, it appears depressed, as shown in Figure 3, *C*. Taken together, these configurations indicate that the peritubular matrix was more deeply etched than the intertubular matrix. Apart from the difference in elevation, the peritubular matrix can easily be distinguished from the intertubular matrix by its characteristic granular appearance. The intertubular matrix is much smoother and shows everywhere a faint pattern of collagen fibrils. A smaller number of fibrils are also observed in the peritubular matrix (Fig. 3, *B*). Some of them seem to run individually, but others form small bundles. The areas between the fibrils are filled with a fine granular substance. When replicas from acid-etched surfaces are subjected to limited-area electron diffraction, an apatite pattern is obtained from the peritubular zone. On the basis of this observation, the granular substance is thought to be inorganic particles liberated from the peritubular matrix during etching and retained on the final replica. That the granules are inorganic is further substantiated by the fact that they are also present in pseudo-replicas of surfaces which have been treated with ethylenediamine subsequent to the acid etch.

As mentioned above, the peritubular matrix appears very clearly on acid-etched dentin surfaces. Stronger acid treatment, however, may destroy it completely. As a result, a channel with a much wider lumen than that of the original dentinal tubule is formed. Figure 3, *C* and *D*, was obtained from specimens which had been prepared, first, by etching a polished dentin surface for 5 seconds with a 0.01 N HCl and then etching half of it with 0.1 N HCl for 10 seconds more. In the upper half of Figure 3, *C*, the peritubular matrix is clearly visible, while it cannot be distinguished in the lower

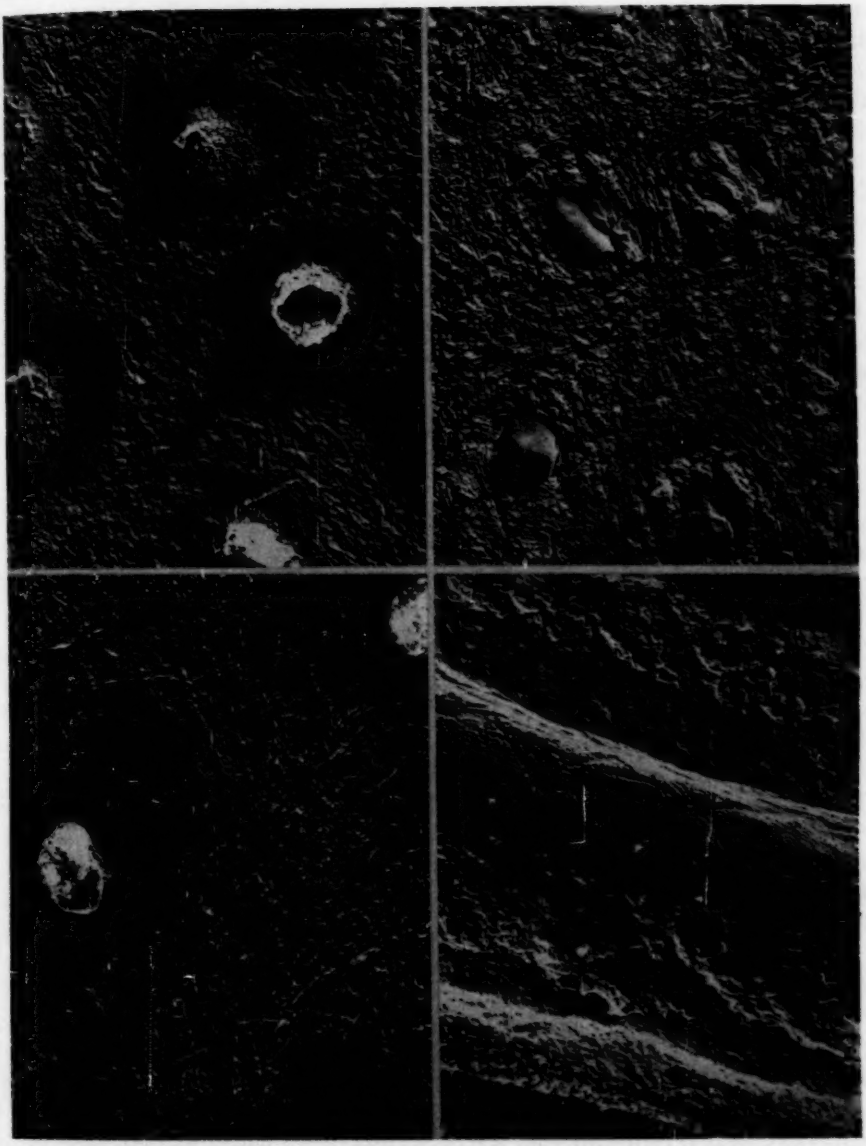

FIG. 2.—Peritubular matrix observed on the surfaces of a polished ground section (*A*), a cleaved fragment of a tooth completely de-organized with ethylenediamine (*B*), a polished ground section treated for 1 hour with ethylenediamine (*C*), and a cleaved fragment of an untreated tooth. (*A*, *B*, *C*, mag. ×6,000; *D*, mag. ×9,000.)

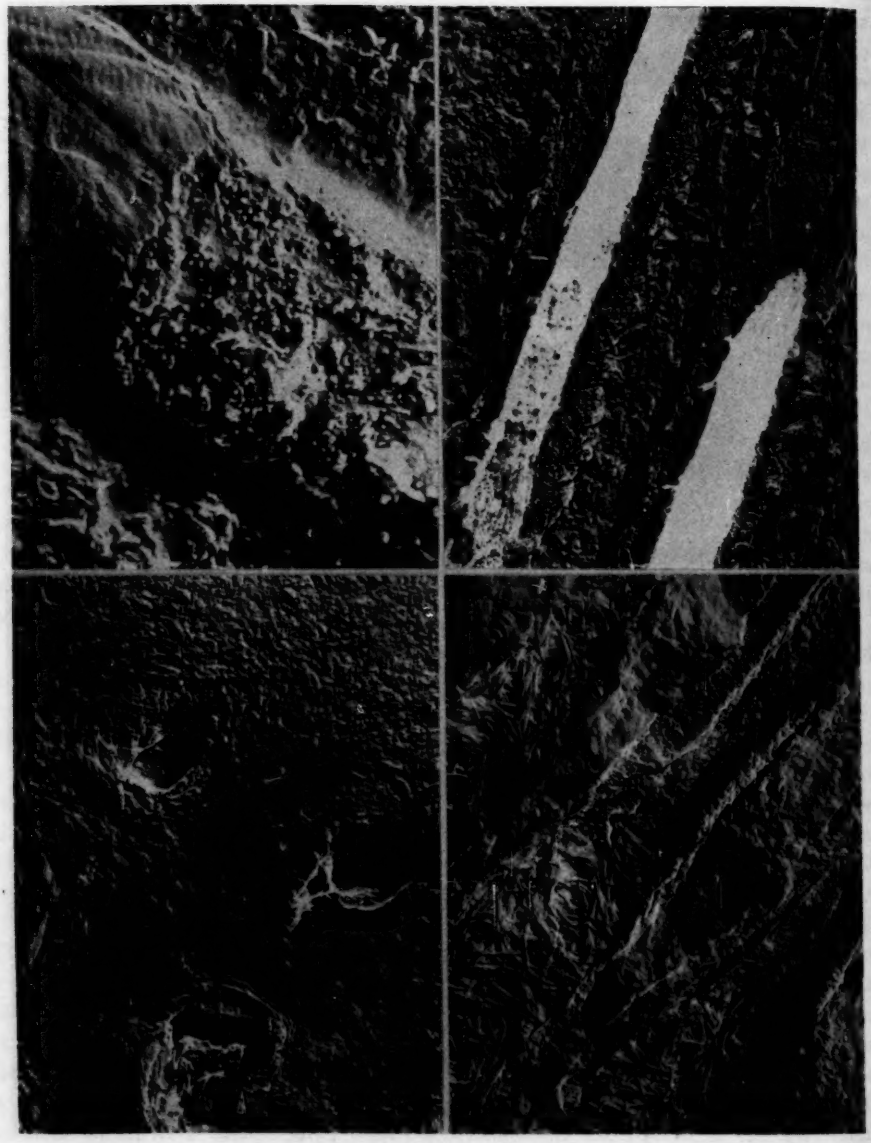

FIG. 3.—*A:* Tubule wall observed in longitudinally cleaved untreated dentin. (Orig. mag. ×20,000.) *B:* Peritubular matrix on an acid-etched longitudinal ground section of dentin. (Etched 5 sec. with N/100 HCl; orig. mag. ×6,100.) *C* and *D:* Destruction of peritubular matrix through stronger acid treatment. Upper right portions of fields shown were etched 5 sec. with N/100 HCl, and those in lower left were treated 10 sec. more with N/10 HCl. (Mag. ×6,100.)

half, which was more deeply etched. The dentinal tubules shown in Figure 3, *D*, were cut longitudinally, and numerous collagen fibrils can be seen in the widened channels.

The six pictures in Figure 4 were taken from different regions spaced at equidistant intervals from the predentino-dentinal border to the dentino-enamel junction. It is evident that the widths of the dentinal tubule and the peritubular matrix change from the predentinal border outward. While the tubules generally decrease in diameter toward the enamel, the peritubular matrix, which appears to be almost absent close to the predentin, gradually becomes wider, to reach maximum thickness somewhere in the middle of the dentin proper. From that point on, however, it decreases again in size toward the dentino-enamel junction.

In addition, a difference is seen in the structure of the peritubular matrix in the different regions. Next to the predentin the texture is much smoother than closer to the enamel, where it appears granular. There is also some indication, on acid-etched dentin surfaces, that the intertubular collagen fibrils in the dentin matrix are more prominent near the dentino-enamel junction than near the predentin.

DISCUSSION

As briefly mentioned in the introduction, there has been more or less definite evidence in recent publications that the odontoblastic process is surrounded by a special structure. Bergman and Engfeldt[17] in their paper, however, raised the question as to whether or not this structure might be a secondary formation caused by posteruptive influences. The finding in the present study that the peritubular matrix is present in unerupted teeth indicates that the structure does not have its origin posteruptively.

With respect to the nature of this structure, Menke[3] and Band and Held[19] reported it as a hypocalcified area, in contrast with most other investigators, who claimed that it was more calcified than the rest of the matrix. Through the present work it is evident that the inorganic component is usually present in higher amounts in the peritubular matrix than in the intertubular. This is shown by the fact that (1) the peritubular matrix appears as a solid and more compact structure than the rest of the matrix on either polished or mechanically cleaved dentin surfaces; (2) it shows a higher resistance to the action of the protein solvent than does the intertubular matrix; and (3) it is more deeply etched by the acid than the rest of the matrix and is more readily destroyed to completeness during strong acid treatment.

Takuma and others,[24, 25] Frank,[10a, 20] and Yamaguchi,[26] in studies of demineralized dentin sections have pointed out that in some instances the peritubular space is filled with submicroscopic fibrillar material. These fine fibrils are thought to be the organic constituents of the peritubular matrix which have remained by chance after removal of the inorganic constituents. This observation suggests that, in spite of being hypercalcified, the peritubular matrix still contains organic constituents in various quantities. According to the present studies, the visible organic structure in the peritubular matrix appears to be composed mainly of collagen fibrils which are probably continuous with fibrils of the same type in the intertubular matrix. The collagen fibrils in the peritubular matrix, however, follow more irregular courses and seem more widely separated than those in the intertubular matrix.

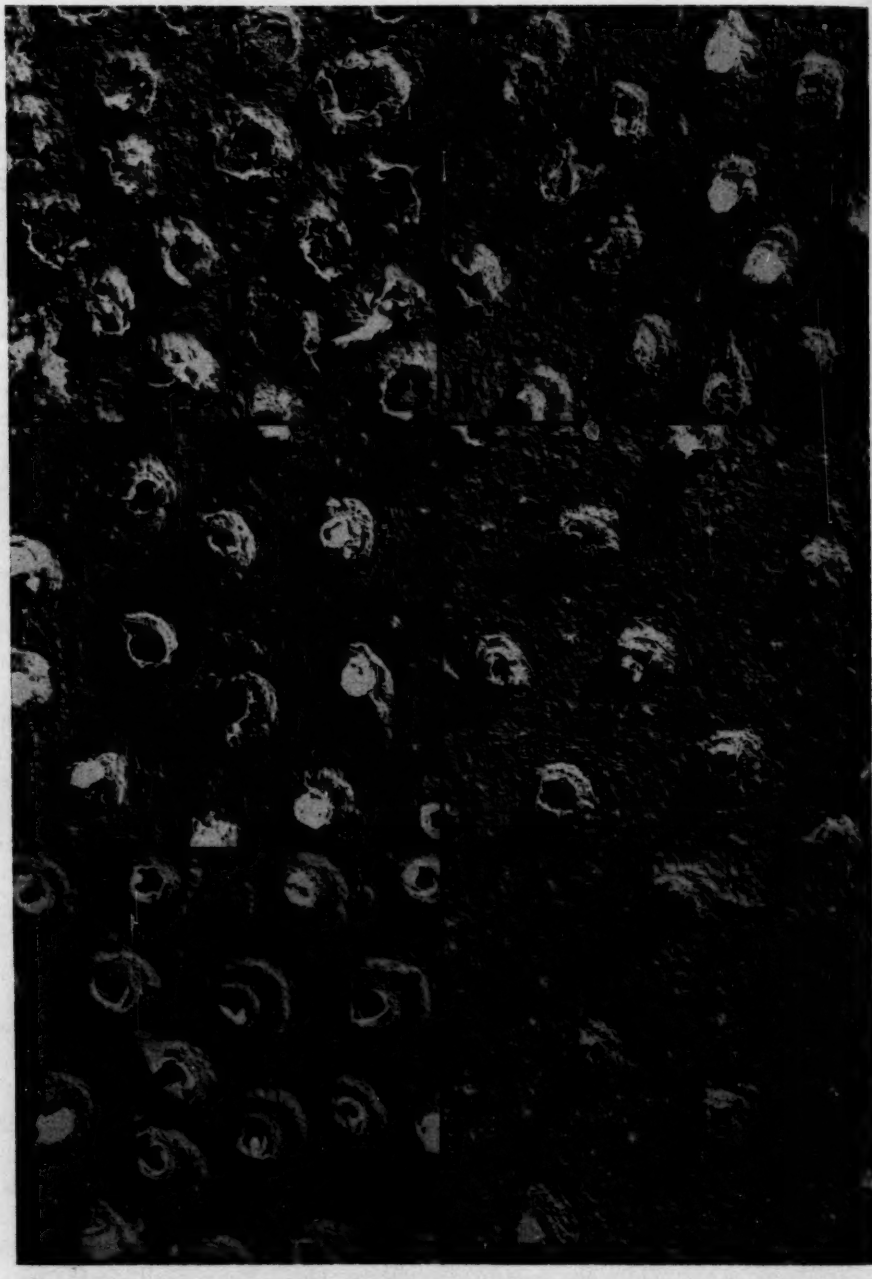

FIG. 4.—Variation in the appearance and dimensions of the peritubular region from the predentin-dentin border to the dentino-enamel junction (*F*) in the same tooth. (Etched 5 sec. with N/100 HCl; mag. ×3,000.)

SUMMARY

The existence and the structure of highly mineralized areas encircling the dentinal tubules have been demonstrated under the electron microscope through the use of improved replica technics. Variation in the appearance and dimension of this special structure has also been discussed.

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*Methods and Techniques in the Preparation and Microradiography of Mineralized Tissues**

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A number of different methods are now in use for the preparation of ground specimens of bone and teeth. Hammarlund-Essler[1] and Sognnaes[2] have described methods in which the specimen is imbedded in methyl-methacrylate, cut into thin slices, then ground on various abrasive papers, and finally polished on emery paper. Roofe, Hoecker, and Voorhees[3] introduced the use of a brain microtome with a saw attachment. This method necessitates imbedding the specimen in bioplastic. These techniques were found difficult to apply when many serial sections were to be studied by microradiography and later stained for microscopic examination. In addition, only a limited number of serial sections could be obtained. Using a cutter of simple design, it was found that serial sections of mineralized tissue 20–100 μ in thickness could be easily obtained in a short time with satisfactory results. The versatility of the cutting equipment also enables the operator to select any desired angle of cut. This paper describes subsequent processing procedures whereby microradiographs of high resolution can be obtained with minimum effort and equipment. The microradiographic technique is a modification of that described previously by Istock, Miller, and Losee.[4]

MATERIALS AND METHODS

Prior to cutting, fixed or fresh bones and teeth are freed of all external soft tissue. No other preparation is required. Initial cuts of approximately 100 μ in thickness are made with the cutter illustrated in Figure 1. An electric motor with governed speeds of 5,000–45,000 rpm is mounted on a standard milling-machine base. The micrometer control permits precise selection of specimen thickness. Initial cuts as low as 50 μ have been obtained. Steel blades 3.5 inches in diameter and 0.010 inch thick, with 48 teeth to the inch, are used. The cutting edge of the blade is cooled by a continuous stream of cold water, thus heat production and shattering of the specimen are minimized.

The specimen holder (Fig. 2) can be rotated 360° horizontally and approximately 120° vertically, thus enabling the operator to select different angles for cross, longitudinal, and oblique sections.

When cutting bone, speeds of 20,000 rpm are used. No apparent advantage is gained by increasing the rate. At this speed, the cutting time of normal cortical bone 2 cm. thick requires approximately 10–12 minutes.

For cutting serial tooth sections, 75–100 μ in thickness, it was necessary to reduce the speed to 6,000 rpm. Cutting time was increased to 20 minutes per section.

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* The opinions or assertions contained herein are the private ones of the writers and are not to be construed as official or reflecting the views of the Navy Department or the naval service at large.

After cutting, the specimens are hand-lapped on the abrasive surfaces of frosted plate glass and moistened with 95 per cent ethyl alcohol. Lapping procedures reduce the sections in thickness and remove minute scratches and scars from the surface of the sections. Specimens are then stored in 95 per cent ethyl alcohol until ready for use.

Sections selected for microradiography are removed from alcohol and air-dried for approximately 10 minutes. The dried specimen is then placed in direct contact with the emulsion and covered with 0.00045 inch of aluminum. The covered packet is

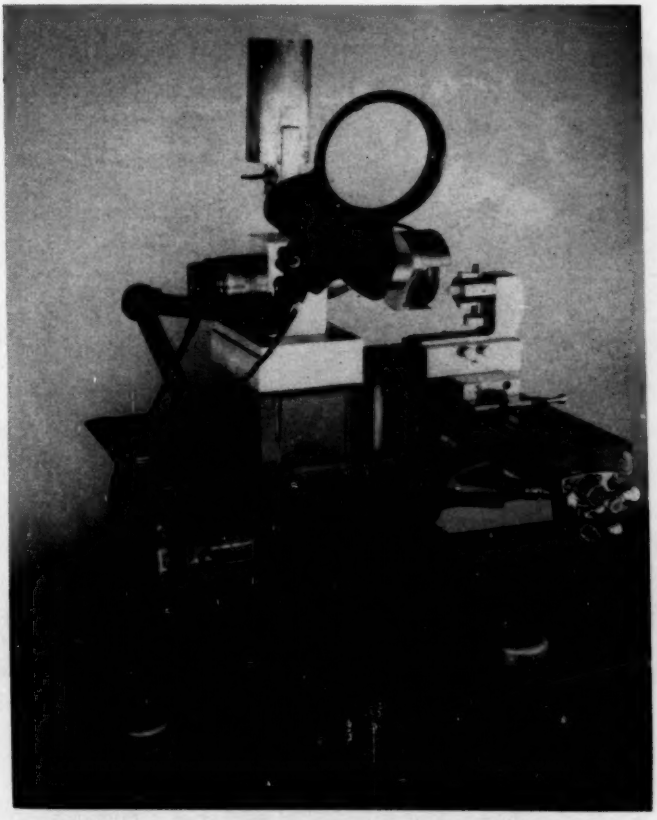

FIG. 1.—A high-speed tooth cutter having a universal motor with speeds ranging from 5,000 to 45,000 rpm. The motor and saw are mounted on a standard milling-machine base.

placed on target 10 cm. from the beam window. For specimens of bone 70μ in thickness, the exposure values are 11 kvp, 45 milliamperes, for 12 minutes. The X-ray apparatus used is a standard commercial generator equipment with a beryllium window tube.* It is a compact unit with a working range of from 5.5 to 50 kvp and current range of from 0 to 60 milliamperes. The unit is water-cooled and designed for continuous operation.

The selected film† has a resolution of approximately one thousand lines per milli-

* Machlett OEG-60.

† Eastman Kodak Spectroscopic Plate 649 GH.

meter. After exposure, the spectroscopic plate is developed* in a high resolution developer at 68° F. for 7 minutes, then placed in an acid fix. The process is completed by washing in running water for 5 minutes, then drying in a dust-free atmosphere.

The complete microradiographs may be stored in a manner similar to that used for routine histologic slides. The slide box should be dust-free. Cover slips are not placed over the image; however, the emulsion may be freed of dust and lint through the use of a soft camel's-hair brush. Photomicrographs can be made by the use of standard photomicrographic material.

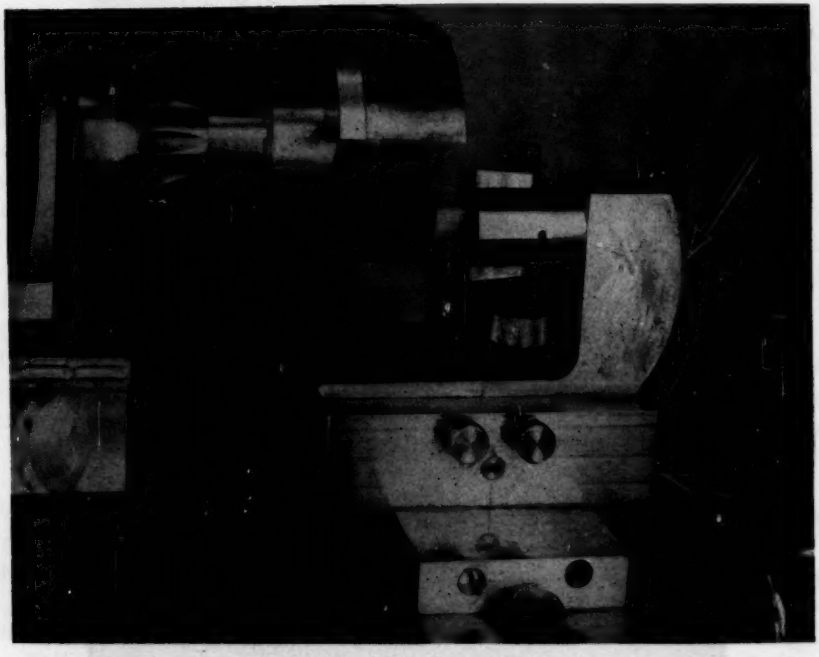

FIG. 2.—A close-up view of the saw and specimen-holding chuck. The base *A* rotates 360° horizontally, and chuck *B* rotates 120° vertically.

RESULTS

Figure 3 is a microradiograph of a 70μ cross-section of a human fibula taken from a sixty-six-year-old male with arteriosclerosis. Varying degrees of mineralization of individual osteons are apparent. Areas of lesser density appear dark. Marrow-vascular spaces and the lacunae appear black. Figure 4 is a microradiogram of a human fibula, showing an individual osteon. Rings of increased mineralization appearing from the lumen toward the periphery of the osteon may be associated with the cement line.

Figures 5 and 6 are microradiogrphs of a dog bone graft. In Figure 5 the homograft appears in the upper right region and the host bone in the lower left region. Large vascular sinuses in the region uniting host to the graft are evident (callus); the mineral bonding the host to the implant is illustrated. In Figure 6 the implant was

* Eastman Kodak D-19 developer.

FIG. 3.—Microradiograph of a cross-section of human fibula 70 μ in thickness, showing the various degrees of mineralization in the Haversian system. (Orig. mag. ×80.)

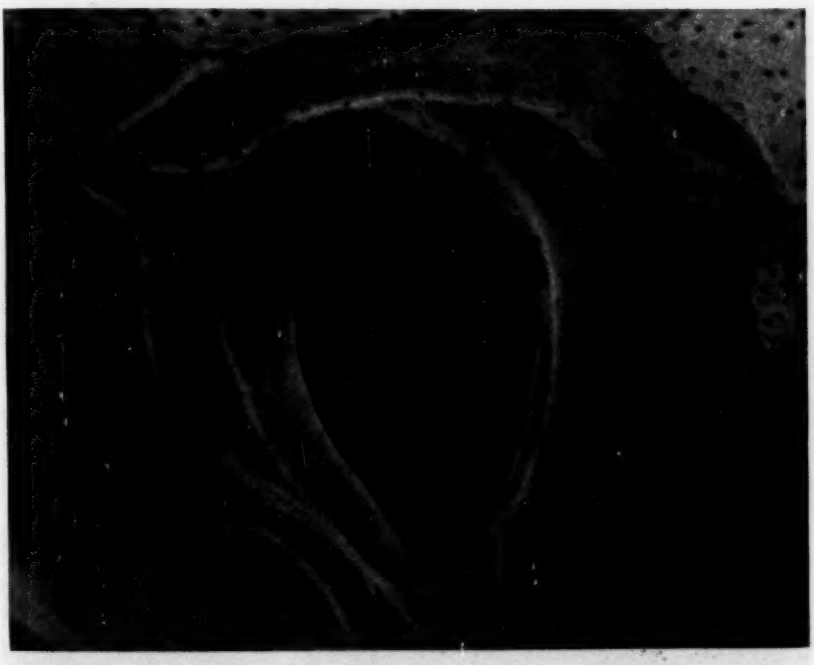

FIG. 4.—Microradiograph of a single osteon. Rings of increased density may be associated with the cement line. (Orig. mag. ×200.)

FIG. 5.—Microradiograph of a dog bone graft 70 μ in thickness. Mineral bonding at the graft site is clearly illustrated. (Orig. mag. ×35.)

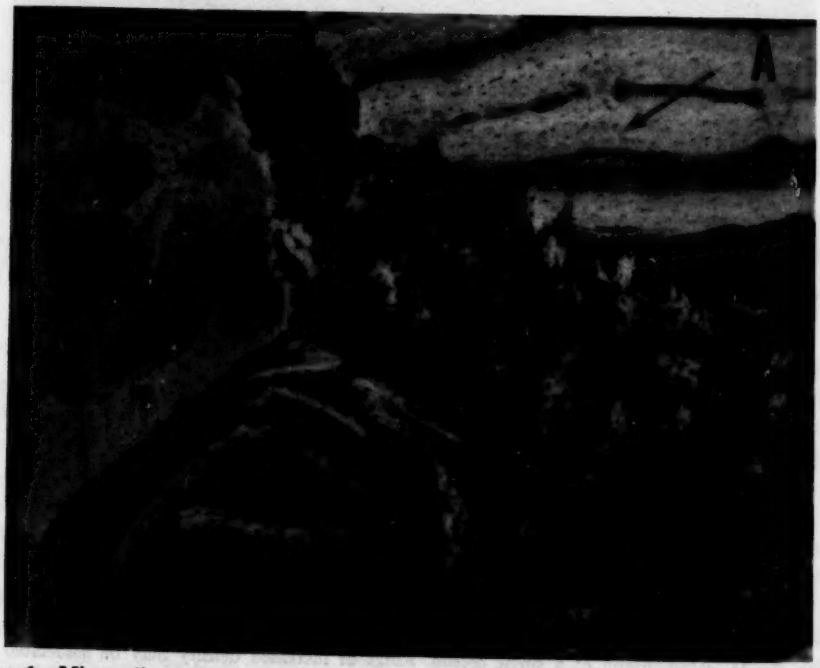

FIG. 6.—Microradiograph of bone graft, 70 μ in thickness. Varying degrees of mineralization are noted at the graft site. (Orig. mag. ×35.)

FIG. 7.—Microradiograph of an early caries, 90 μ in thickness. At the caries site an area of demineralization is noted. (Orig. mag. ×34.)

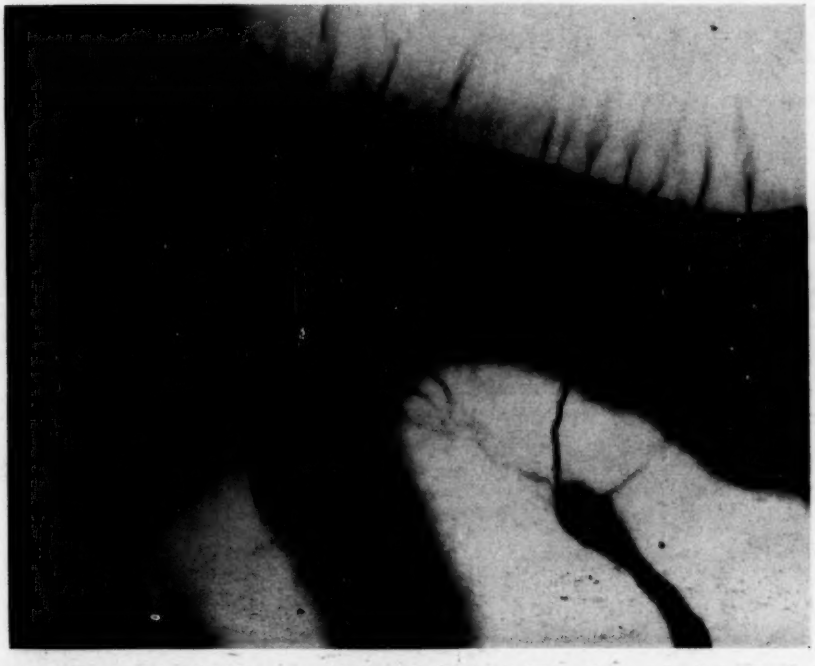

FIG. 8.—Microradiograph of human tooth, 90 μ in thickness. The tufts are clearly outlined at the dentino-enamel junction. (Orig. mag. ×34.)

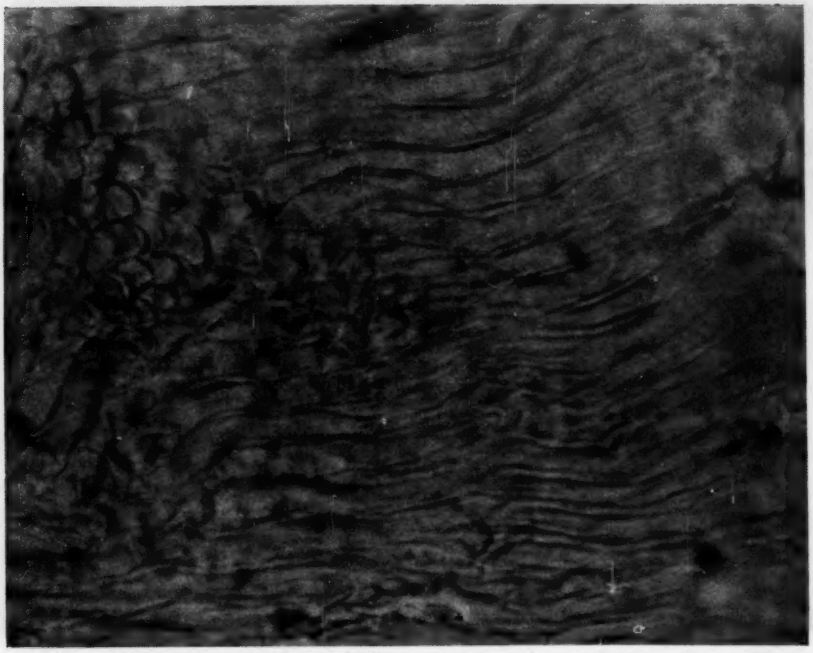

FIG. 9.—Photomicrograph of a ground section of a human tooth, 20 μ in thickness. In the enamel the prisms are clearly visible. (Orig. mag. $\times$400.)

FIG. 10.—Photomicrograph of an unstained ground section of human cortical bone, 20 μ in thickness. (Orig. mag. $\times$80.)

placed in such a manner as to present the Haversian channels in cross-section (*a*).

Figures 7 and 8 are microradiographs of human tooth, 90μ in thickness. In Figure 7 a carious lesion in the enamel illustrates clearly an area of decreased mineralization at the caries site. After the crown of the tooth was removed, the following first section was radiographed in Figure 8. The dentin, being less dense than the enamel, appears as a dark area. At the dentino-enamel junction, tufts are clearly visible.

Figure 9 is a photomicrograph ($\times$ 400) of a ground section of a human tooth, 20μ in thickness. In the enamel the prisms are clearly visible. Figure 10 is a photomicrograph of human cortical bone, cut ground section, 20μ in thickness, showing several Haversian systems. Microcanaliculae extending from osteocyte lacunae are clearly visible. The cellular components of the central canal are present.

SUMMARY

A relatively simple but rapid method for preparing ground sections of mineralized tissues for use in microradiography is described. Sections less than 50μ in thickness can easily be obtained in approximately 30 minutes. Sections 100μ or less in thickness can be examined by visible light or studied by microradiography. The differences in mineral deposition can be easily established.

Special mention is given to B. Goldstein, HM1, U.S.N., and H. T. Dent, HM3, U.S.N., for assistance in the cutting and lapping of the bone and teeth used in this report.

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Methods of Preparing Stained Ground Sections of Soft and Hard Tissues

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In dental research the use of ground sections is one of the oldest methods. However, during the process of grinding, all the soft tissues are lost. During routine microtomy of histologic specimens, on the other hand, which involves decalcification, the enamel is lost, and normal relations are destroyed. Thus, until recently, we have not had at our disposal a satisfactory method for the preparation of tissues for microscopic study which would simultaneously preserve soft and highly calcified structures in their normal relationships and composition. Previous methods suffered from the inadequacy of the usual imbedding materials, which were brittle and lacked the necessary tensile strength. This deficiency has been overcome by the use of plastics as infiltration media. In this paper a brief description is given of some newer methods developed or adapted in our laboratory.

The ingenious principle of Koch's[1, 2] petrification technique—originally designed for preparing sections of highly calcified corals—was first applied to dental structures by Weil[3, 4] and later modified by Mayer,[5] Chase,[6] and others.

The advent of plastics made possible further progress in this field. Sognnaes[7] used methyl methacrylate infiltration for the sectioning of teeth and jaws, while Lacroix[8] and Kropp[9] applied this method to the cutting of bone.

The method of preparing the sections depends on the equipment available. Meyer,[5] Gustafson,[10] and Orvig[11] recommend manual grinding on glass plates with abrasive powder in liquid. Hand grinding may also be done on rotating disks.[7, 12, 13] Many laboratories possess wholly mechanical devices for sectioning and grinding.[14–22]

MATERIALS AND METHODS

Human teeth, as well as jaws from monkeys, dogs, cats, and rats, were imbedded and cut. Two plastics were used for imbedding: (*a*) methyl methacrylate monomer,* which is obtainable commercially for the processing of acrylic dentures, and (*b*) a polyester resin,† which is supplied as a clear, viscous liquid. From the former plastic the various solutions were prepared according to Sognnaes.[7] This plastic exhibits a great deal of contraction during polymerization and a tendency toward bubble formation. It can also be at least partially dissolved by its own monomer. The polyester resin is freely miscible with methyl salicylate for reducing its viscosity for better infiltration. According to the manufacturer, there is no known solvent for this resin.

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* Krystolex, The Kerr Co., Detroit, Mich.

† Bioplastic, Ward's Natural Science Establishment, Inc., Rochester, N.Y.

The formalin-fixed material was well washed under running water for 24 hours and then passed through ascending grades of ethyl alcohol for dehydration. If destined to be imbedded in methyl methacrylate, it was then cleared in xylol. Methyl salicylate was used for specimens to be imbedded in polyester resin.

A. *Methyl methacrylate.*—Imbedding with this material was much the same as that recommended by Pucket[23] for commercial and general biological uses of plastic materials and as applied to dental structures by Sognnaes.[7]

B. *Polyester resin.*—This plastic was processed in accordance with the manufacturer's recommendations for the imbedding of anatomic specimens. However, for better infiltration, two intermediary solutions consisting of mixtures of polyester resin and methyl salicylate were used. Imbedding under vacuum helped to speed up the process considerably.

C. *Double imbedding.*—In order to combine the relative merits of both methyl methacrylate and polyester resin, a method of double imbedding—analogous to the celloidin-paraffin method—was developed. The material to be sectioned is dehydrated in alcohol, cleared in xylol, and infiltrated first with methyl methacrylate, while final imbedding is done in polyester resin. In this method the tissues are infiltrated with methyl methacrylate, a material for which solvents exist. When deemed necessary, the plastic can be either partially or completely removed from them, while the whole section is still held together by its outer frame of polyester resin. Furthermore, blocking is much easier and is not accompanied by the great contraction of the methyl methacrylate and its tendency toward bubble formation.

We have used both manual and mechanical preparation of sections with good results.

Both staining of the specimen *in toto* previous to infiltration and staining of the unmounted ground sections were tried. Staining *in toto* was done in accordance with routine histologic technique, before imbedding. However, the thickness of the specimen, as well as the coexistence of soft and very hard tissues, made uniform penetration of the dyes impossible, and hence this method gave inconsistent results. Staining of the ground sections is also similar to the usual histologic method, but it requires more time. Usually only the two surfaces of the section are stained. Deep penetration of the dye occurs only where plastic infiltration has been insufficient. Thus images of stained tissue can be obtained under the microscope at two different focal planes. Focusing, therefore, has to be done carefully, in order to avoid a disturbing overlapping of the two views.

If the dyes used are waterproof, the undesired stain can be removed from one surface by repolishing. A way of staining only one surface is to fix the section to the slide with a cement impervious to the dye; on being stained, only the free surface will absorb the dye; in either instance, only the superficial layer of these relatively thick sections is stained, and the tissues can be examined at high magnification, even with oil-immersion objectives.

The following stains were employed:

A. *Hematoxylin and eosin.*—In our hands, ordinary H & E staining did not prove compatible with plastic imbedding. (Good results were obtained only with Regaud's and Harris' hematoxylin, but even these were not consistently good.)

B. *Methylene blue* (*Figs. 1–6*).—We found this to be the best nuclear stain. It can be used alone or in combination with other stains. Good nuclear and general

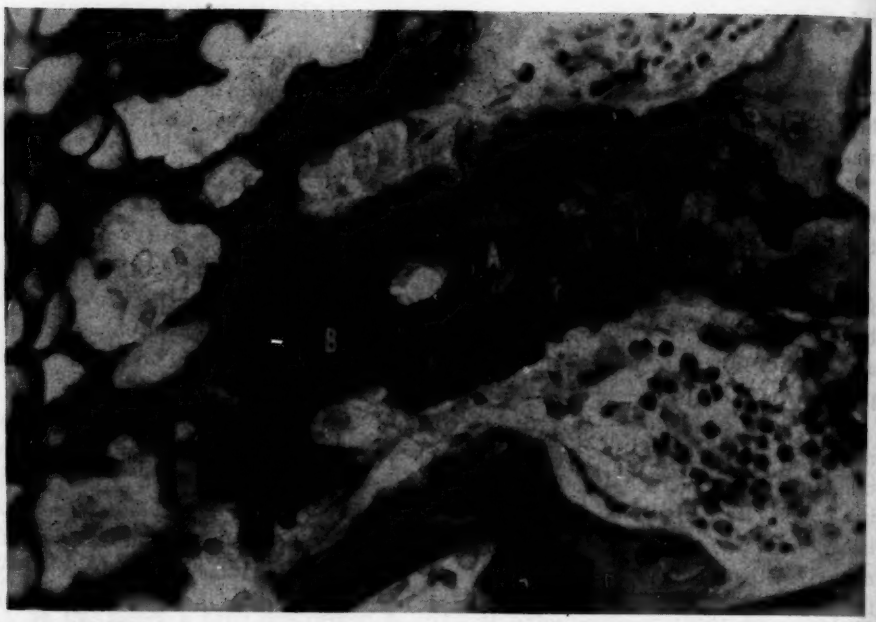

FIG. 1.—Microphotograph of ground section of rat femur showing bone development near the metaphysis. Newly formed bone (*A*) is seen surrounding the remnants of calcified cartilage (*B*). Note preservation of cells in bone marrow (*C*). (Methylene blue; orig. mag. ×430.)

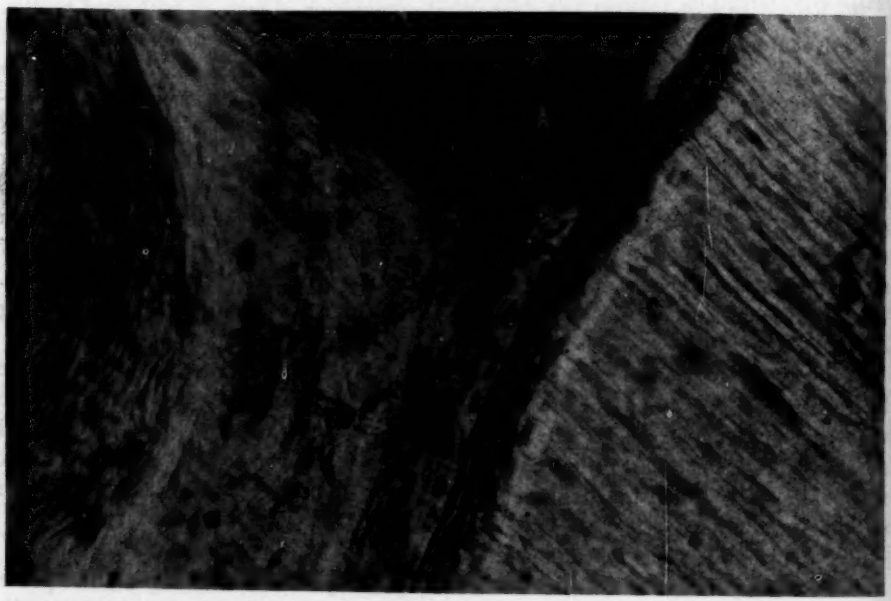

FIG. 2.—Microphotograph of ground section of incisor of monkey with its surrounding tissues. A foreign material (*A*) was introduced into the gingival sulcus, in order to demonstrate the existence of an epithelial attachment. The epithelial cells (*B*) can be seen clearly in a continuous layer under the foreign material to their firm union with the enamel of the tooth (*C*). (Methylene blue; orig. mag. ×430.)

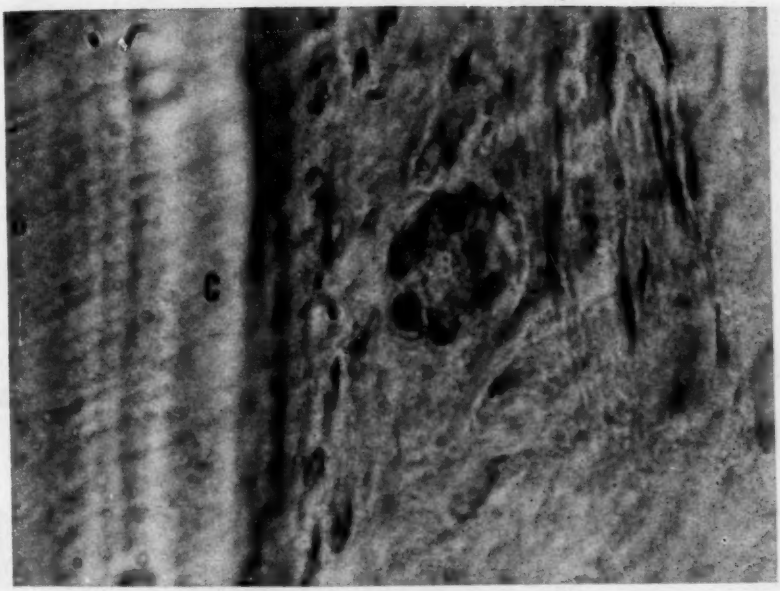

FIG. 3.—Microphotograph of ground section of periodontal membrane (*A*) of monkey molar. At *B* are epithelial remnants near the cementum (*C*) and cementoblasts (*D*). (Methylene blue; orig. mag. ×970.)

FIG. 4.—Microphotograph of incisor of monkey, demonstrating ameloblasts at *A*. (Methylene blue; orig. mag. ×430.)

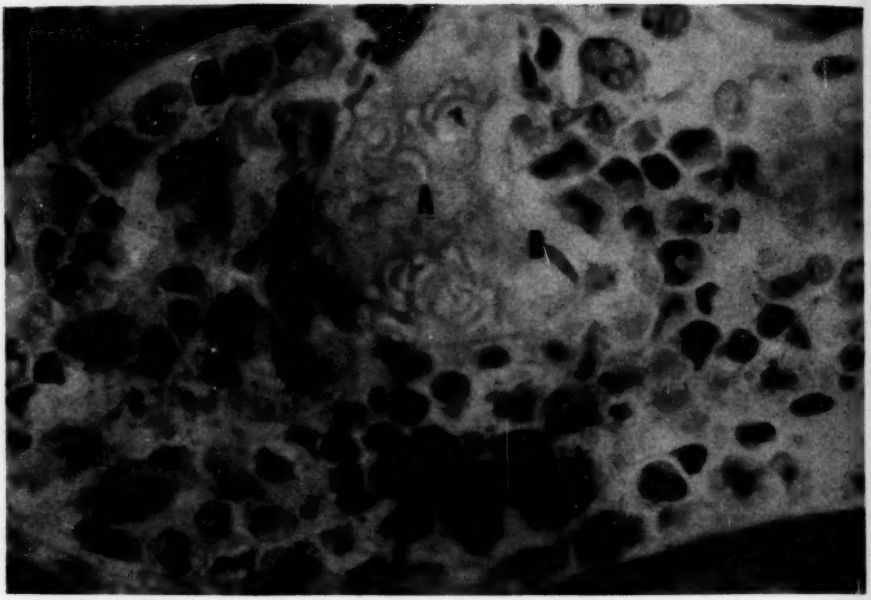

FIG. 5.—Microphotograph of ground section of rat femur. High-power view of an area of bone marrow, showing capillary at *A*, endothelial cell at *B*, and details of cells of the marrow (*C*). (Methylene blue; orig. mag. ×970.)

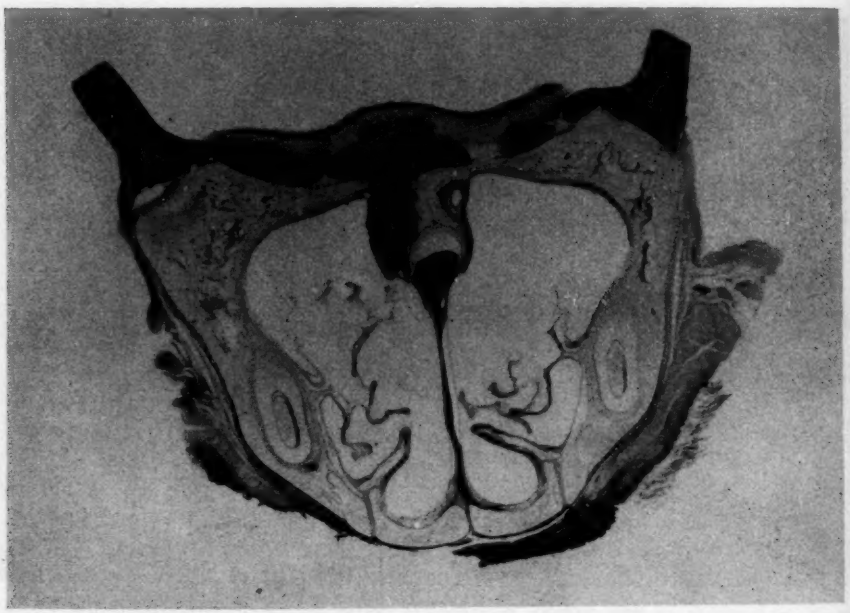

FIG. 6.—Photograph of ground section of maxilla of dog, showing an experimental metallic implant. (Methylene blue.)

staining can be obtained in 20 minutes with a 0.5 per cent aqueous solution. We sometimes counterstained with a concentrated solution of eosin in absolute alcohol for 10 minutes.

C. *Giemsa's stain.*—This also gave good nuclear and general staining, but it had to be used with care to avoid precipitation. We used the concentrated stain that is normally diluted for blood smears. The section was washed in distilled water, stained for 3–5 minutes, and washed again. After blotting on filter paper and drying, it was directly transferred to xylol.

D. *Mallory's connective tissue stain.*—Crossmon's[24] modification of Mallory's connective tissue stain also gave good results but insufficient nuclear staining.

Methyl methacrylate–imbedded tissues were more difficult to stain unless some of the plastic was superficially dissolved. This, however, endangers the soft tissues.[25] It was thought that the double-imbedding method would overcome this difficulty. However, no appreciable difference could be noted in the staining properties of these double-imbedded sections.

SUMMARY

Methods for processing simultaneously both soft and hard tissues for histologic study are described. A commercial polyester resin and methyl methacrylate were used for imbedding, either individually or in a double-imbedding technique. Manual or mechanical grinding was employed for obtaining single or serial sections. The various staining techniques applicable to these sections are given, and the results obtained are illustrated.

The authors wish to express their sincerest thanks to Professor M. Iczkowicz, head of the department, for his constant encouragement and advice, to Dr. G. Gitlin for his helpful suggestions, and to Mrs. C. Salomon for her excellent photography.

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*Relation of the Adrenal Cortex to the Salivary Secretion Mechanism of the Dog**

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Ludwig[1] in 1851 showed that the normal activity of salivary glands is under nervous control, by which there is reflex stimulation of saliva secretion. Heidenhain,[2] the first to clarify the secretory mechanism for salivary glands, accepted both nervous and humoral stimulation. Later, Babkin[3] proposed that cellular permeability and the metabolism of the glands might be subject to both nervous and humoral stimulation; yet saliva is still generally considered the only digestive secretion dependent solely on a nervous regulation.

Because of the importance of this problem, we have examined the role of the adrenal cortex in salivary gland secretion, producing gradual corticoadrenal atrophy in dogs by administering the substance 2,2-bis(parachloro-phenil)-1,1-dichloroethane (DDD†), according to the method of Nelson and Woodard.[4, 5] In such animals we have studied (1) the changes in weight and the histologic alterations in the submaxillary gland, (2) the variations in salivary flow, and (3) the protective effect of the adrenal cortical hormone.

EXPERIMENTAL METHODS

Forty-three male or female dogs, weighing between 5.6 and 15.0 kg each, were divided into 3 groups: (1) twelve control and thirteen DDD-treated dogs, used for observations on weight and histologic changes in adrenal and submaxillary glands; (2) six control and six DDD-treated dogs, used for quantitative determinations of salivary flow; and (3) six dogs simultaneously receiving DDD and corticoids, used for observations on the effectiveness of corticoids in protecting the dogs against the weight changes, histologic alterations, and decreases in salivary flow previously investigated in groups 1 and 2. All animals were confined in individual cages and checked daily for change in weight, food consumption, and general behavior.

DDD was given orally in gelatin capsules in doses of 100 mg/kg/day for periods of 7–27 days, depending on the clinical condition of the animals. As a rule, DDD administration was interrupted when the animals showed a drop in weight, anorexia, or blood diarrhea. The DDD-corticoid group received simultaneously the DDD during

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† Rhothane, manufactured by Rohm & Haas Co. Laboratories, Philadelphia, Pa.

a period of 21–27 days and an adrenal cortical extract (free of epinephrine*) corresponding to 25 gm. of fresh gland (50 dog U) daily intramuscularly.

To determine the salivary flow, each dog was anesthetized with sodium pentobarbital intravenously (30 mg/kg). The Wharton duct was canulated with a polyethylene tube. The rate of flow was determined for 5- and 30-minute periods, injecting pilocarpine intravenously (0.5 mg/kg in 2 ml. saline). To protect against asphyxia from the large volume of saliva, intratracheal intubation was done in every case.

At the end of the experiments, both adrenals and the left salivary gland were removed, carefully cleaned, weighed (with an approximate error of 1 mg.), and preserved for histologic studies.

RESULTS

Glandular weights (Table 1) are expressed as mg/kg body weight, to minimize the effect of variation in size of the animals. On this basis, the adrenals of the DDD-treated and DDD-corticoid–treated groups were reduced in weight by 36.6 and 48.3

TABLE 1

WEIGHT ALTERATIONS OF LEFT SUBMAXILLARY AND BOTH ADRENAL GLANDS OF CONTROLS AND DDD-TREATED DOGS

Treatment	No. of Dogs	Dog Weight (Kg.)	Weight of Both Adrenals (Mg/kg)	Weight of Left Submaxillary (Mg/Kg)	Treatment Period (Days)
Control.....	12	10.2±0.7*	145±5.5	575±32.5	.....
DDD.....	13	8.0±0.5	92±7.9	398±16.2	7–27†

* Standard error of the mean.

† Minimum and maximum periods.

per cent, as compared with the controls. The weights progressively decreased as the period of treatment increased. Since the DDD-corticoid group received DDD for a longer period, on the average (21–27 days), than did the DDD group (7–27 days), this may explain why the adrenal weights for the DDD-corticoid group were lower.

The weights of the submaxillary glands (mg/kg) were reduced by 31.0 per cent in the DDD-treated and 26.0 per cent in the DDD-corticoid–treated group. The reductions in weight of adrenal and submaxillary gland were highly significant statistically ($P < 0.001$). There was a close correlation between reduction in adrenal weights (Fig. 1). The adrenal cortical extract did not protect against the glandular weight loss caused by DDD.

Histologic observations have been published elsewhere,[6, 7] and only new findings in the submaxillary gland will be summarized here. Two principal types of lesions were observed. The first consisted in atrophy of the glandular acini and of the intralobular canaliculi, with nuclear pyknosis. Despite the parallel evolution with the adrenal changes, such lesions were restricted to certain regions of the gland only. The adrenal corticoids did not protect against these lesions. The second type of lesion found in the submaxillary gland consisted of generalized vascular changes, with a variable de-

* Slocort, manufactured by Industria Farmaceutica Endochimica, Brazil, S.A.

gree of thrombosis, deposit of pigment, and hyaline degeneration. These vascular changes were not found in the group treated with both DDD and corticoids.

The maximum salivary flow during pilocarpine stimulation (Table 2) was reduced by 82.0 per cent in the DDD-treated, as compared with the control group. In the DDD-corticoid group the mean rate of flow was only 18.0 per cent below the control. The reduction in the DDD-treated group was statistically highly significant; that in the DDD-corticoid–treated group was not. The period of maximum salivary flow was also shorter in the DDD-treated than in the DDD-corticoid–treated group; in the former we never observed a salivary flow lasting longer than 5 minutes. Under the combined DDD-corticoid treatment the period of flow was always more than 30 minutes and the volume sometimes as great as 50 ml.

DISCUSSION

As Langley[8] demonstrated in 1901, the adrenal hormones increase salivary secretion. In 1920, Hämmerli[9] observed in a man at autopsy a striking hypertrophy of the pa-

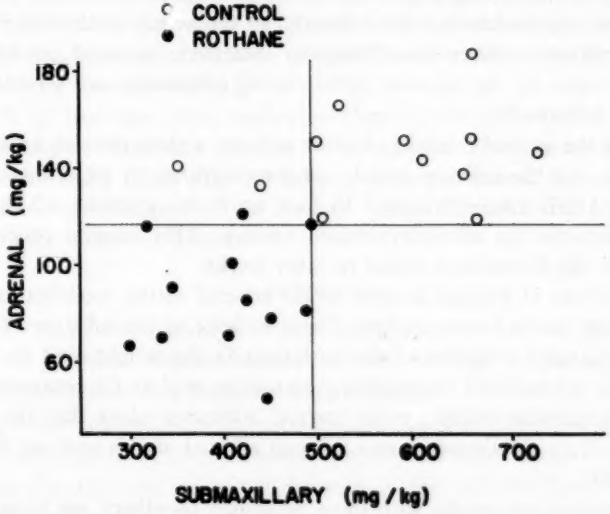

FIG. 1.—Correlation of adrenal and submaxillary gland weights (mg/kg) between controls and DDD-treated dogs.

TABLE 2

SALIVARY FLOW IN 5-MINUTE PERIOD AFTER 0.5 MG/KG PILOCARPINE ADMINISTRATION IN CONTROLS, DDD-TREATED, AND DDD-CORTICOID-TREATED DOGS

Treatment	No. of Dogs	Dog Weight (Kg.)	Weight of Left Submaxillary (Mg/Kg)	Flow	Treatment Period (Days)
Control	6	12.0±1.5*	521±47.4	5.6±0.4	.....
DDD	6	9.4±0.8	369±17.6	1.0±0.3	12–27
DDD and corticoids	6	7.9±0.7	426±22.1	4.6±0.9	21–27

* Standard error of the mean.

rotid and submaxillary glands associated with hypertrophy of the adrenal glands. After these two basic observations had been made, no further experiments were conducted for the purpose of evaluating the role of different hormones in the salivary secretion mechanism. Therefore, no paper relating to adrenal and salivary glands appeared until 1946.

In castrated and adrenalectomized mice, Raynaud [10, 11] observed that the effect of both operations produced a more pronounced atrophy of the submaxillary serous tubules than did castration alone. This was explained on the assumption that corticoadrenal hormones also can stimulate the serous tubules to some extent. However, the data of this author indicated that the role of the adrenal was only slight, if any.

Understanding of the action of adrenals upon the salivary glands has increased since 1955. Bixler *et al.*[12, 13] observed that the adrenal glands of rats from which the salivary glands had been removed exhibited an increased lipoid activity and a marked cellular hypertrophy of the fasciculata. In adrenalectomized cats the submaxillary glands may be strikingly reduced, with slight effect of DOCA in preventing the atrophy.[14] A histochemical study of the salivary glands in cortisone-injected white mice resulted in an increase in the polysaccharide color intensity on the acinar epithelium.[15] The changes observed in salivary sodium concentrations indicated increased production of salt-regulating hormone by the adrenal cortex during pregnancy and especially in the severe forms of toxemia.[16]

Contrary to the general thinking of some authors, a close relation exists between the adrenal cortex and the salivary glands, as demonstrated by experimental atrophy of adrenals with DDD administration.[6] In fact, until this moment, all the experiments have been conducted on adrenalectomized animals. This surgical process is possibly responsible for the discordance found in other works.

Under conditions of gradual atrophy of the adrenal cortex, modifications of the submaxillary glands can be better studied. The specificity of the substance for the adrenal cortex,[4, 5] the parallel evolution of the variations in the weight, and the histologic alterations of the adrenal and submaxillary glands, as well as the protection of the salivary flow and vascular lesions by the cortical hormones, show that the modifications in the submaxillary glands are caused through adrenal glands and not directly by the action of DDD.

When comparing our results with those published by others, we found that we had obtained a greater degree of submaxillary gland atrophy. Kahlson and Renvall[14] obtained in adrenalectomized cats a 28.0 per cent reduction in the submaxillary gland weight (mg/kg), while in our DDD-treated dogs we found a 31.0 per cent reduction. However, in both experiments some individuals had a submaxillary gland reduction near 50.0 per cent. Simultaneous administration of corticoids and DDD did not influence the adrenals, while the submaxillary gland weight reduced to 26.0 per cent, suggesting that the amount of corticoids was not great enough to protect the integrity of the whole gland.

The influence of the adrenals on salivary flow was well demonstrated in this work. Some authors pointed out the effect of thyroid dysfunction on salivary flow and viscosity in the rat.[17] In analyzing such results, we can see that propylthyouracil-treated animals showed only a 45.0 per cent reduction in salivary flow, as compared with an 82.0 per cent reduction in DDD-treated animals, which suggests the greater influence

of adrenals on the salivary flow, despite the fact that different species of animals were used.

The principal histologic findings in regard to the submaxillary gland consisted of generalized vascular changes which possibly explain the differences in the reduction of salivary flow and submaxillary gland weight. As demonstrated and published elsewhere,[18] we believe in a direct influence of the adrenal cortex on the submaxillary enzymatic activity in the dog. These results agree with the investigations and ideas of some authors explaining the salivary secretion mechanism, despite the different methods employed.

The vascular importance in the salivatory process, admitted by Langley[19] in the last century, was confirmed in a categorical way by other experiments. The changes in the gland cells, when activated,[20, 21] permit the escape into the interstitial fluid of an intracellular enzyme which acts on the proteins present to form a vasodilator polypeptide. The saliva vasodepressor activity probably originates from the same substance. Because the enzymatic activity responsible for vasodilatation is a basic event in salivary secretion, such a mechanism cannot be an independent phenomenon, as believed by some authors who used atropine.[22] Vasodilatation is reduced but not abolished by this drug. The simultaneous occurrence of vasodilatation and saliva secretion depends on the intensity of stimulation.[23]

Although it is clear that some endocrine glands participate in the mechanism of salivary secretion, we think that the role of the adrenal cortex is more important.

Finally, we presently think that the salivary secretion cannot be considered as being under the exclusive control of the nervous system. The salivary glands are somehow related to the endocrine system.

SUMMARY

In order to study the influence of the adrenal cortex on the salivary secretion mechanism, we produced adrenal cortical atrophy in dogs by the administration of 2,2-bis(parachloro-phenil)-1,1-dichloroethane(DDD).

With progressive adrenal atrophy, parallel changes were observed in the submaxillary gland: a decrease in weight; histologic lesions, including atrophy of the glandular acini and vascular thrombosis; and a decrease in the salivary flow, which could be evoked by pilocarpine. The histologic lesions and the decrease in salivary flow, but not the decline in salivary glandular weight, were, in part, prevented by the concurrent administration of adrenal cortical extract and DDD.

The salivary secretion mechanism is discussed on the basis of adrenal cortex interference in both the glandular and the vascular activities.

Grateful thanks are due to Dr. Hardy Ebling for his histologic study, to Dr. Edgar M. Wagner for statistical assistance, and to Dr. M. E. Krahl of Chicago for help in the preparation of this manuscript.

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The Demonstration of Glycerophosphoric Acid Dimethacrylate in Dentin and Filling Material Following the Use of a Cavity Sealer

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In 1952 Kramer and McLean[1] reported histologic studies which showed that there was some interaction between dentin and the cavity seal liquid supplied for use with a self-polymerizing acrylic resin.† This was indicated by altered staining reactions in the dentin bordering the cavity; in this area the dentin matrix showed an increased affinity for hematoxylin and altered reactions with many other staining techniques. The zone of affected dentin matrix was approximately 3 μ wide, and there was also a similar alteration in the staining reactions of the dentinal fibrils in the affected area.

In 1956 Buonocore, Wileman, and Brudevold,[2] in a series of experiments designed primarily to test the adhesion of various compounds to dentin, found that the same cavity seal liquid, or a mixture of similar composition, adhered more strongly than any other preparation investigated.

These investigations also confirmed the formation of the zone of dentin having altered staining reactions, and in 1958 Buonocore and Quigley[3] extended the observations on the staining reactions of this zone and showed that preliminary superficial or complete decalcification of the dentin with hydrochloric acid increased the width of the zone.

The development of a material that can form a bond with the calcified dental tissues that is both strong and lasting might have many applications in both restorative and preventive dentistry.

In relation to their work on the adhesion of materials to tooth substance, Buonocore *et al.*[2] stated that "more important than any practical clinical aspects at this time is the possibility suggested by our results that a certain chemical structure is required in order to obtain adhesion to dentin. If this point can be established, then compounds can be specifically tailored for the job of adhesion." The purpose of the present communication is to report experiments that indicate the nature of the chemical component of the cavity seal liquid responsible for the zone of dentin giving altered staining reactions and presumably for the adhesion and to report a technique that permits the demonstration of the distribution of this compound both in the dentin to which it has been applied and in the adjacent layer of an overlying filling.

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† "Sevriton" brand self-polymerizing acrylic resin and "Sevriton" Cavity Seal. Amalgamated Dental Company Limited, 26–40 Broadwick Street, London, W. 1.

MATERIALS AND METHODS

The experiments were carried out on human teeth. Some were non-carious premolars from adolescents, others were non-carious or carious teeth from older patients. When carious teeth were used, the cavities were cut in parts of the teeth not involved by caries. The cavities were prepared with steel burs, the dentin was dried with cotton wool without any dehydrating agent, and the various materials were applied as described later. Formalin fixation was in neutralized 5 per cent formol saline. For paraffin sections the teeth were decalcified in 5 per cent trichloracetic acid, washed in running tap water, dehydrated in ascending grades of ethyl alcohol, cleared in benzene, and vacuum-imbedded in paraffin wax. The sections were cut at a setting of 5 μ on a sledge microtome. When frozen sections were required, the teeth, after decalcification and washing, were sectioned on a CO_2 freezing microtome without any supporting medium at an approximate thickness of 10–15 μ. In certain of the experiments, undecalcified sections were prepared on a modified lathe, using carborundum-impregnated vulcanite disks; alternatively, the teeth were cut in half in a plane passing through the cavity, and these half-teeth were used in place of sections, being viewed by incident illumination.

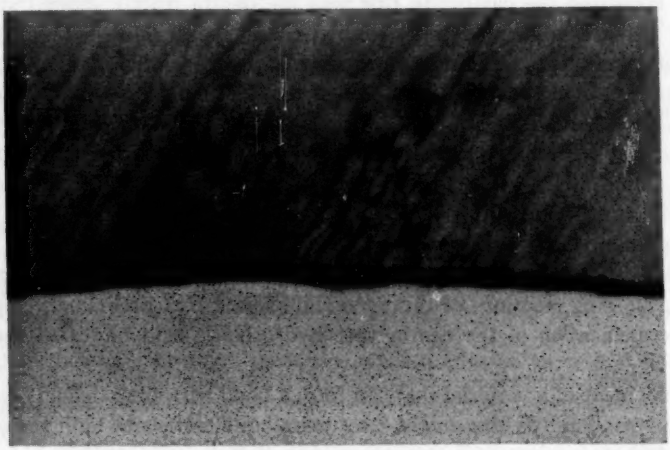

FIG. 1.—Decalcified section of a tooth filled with the resin after the application of cavity seal liquid to the dentin. Next to the cavity there is a narrow zone of dentin staining strongly with hematoxylin. (Hematoxylin and eosin; orig. mag. ×900.)

Hematoxylin staining was with Erhlich's hematoxylin; Schiff's reagent was prepared by the method of De Tomasi.[4]

RESULTS

The present series of experiments arose out of the observation that, in paraffin sections of teeth filled with the resin after the application of the cavity seal liquid to the dentin, the band of dentin that previously had been found to give altered staining reactions (Fig. 1) also gave a strongly positive reaction with Schiff's reagent (Fig. 2) without preliminary hydrolysis or oxidation (direct Schiff reaction). Teeth filled with

the same resin, but without prior application of the cavity seal liquid, gave a negative Schiff reaction, and, as reported previously, there was no zone giving altered staining reactions. It was evident, therefore, that some component of the cavity seal liquid was responsible both for the altered staining reactions and for the direct Schiff reaction. According to the manufacturers, this liquid contains methyl methacrylate monomer, methacrylic acid, and glycerophosphoric acid dimethacrylate. Further experiments showed that the zone of dentin giving altered staining and the direct Schiff reaction could not be obtained when the cavity was treated with methyl methacrylate monomer alone or with methacrylic acid alone prior to the insertion of the resin filling. However, if a cavity was treated with "pure" glycerophosphoric acid dimethacrylate and then filled with the resin, the band of dentin giving both increased affinity for hematoxylin and the direct Schiff reaction was again present. It was clear, there-

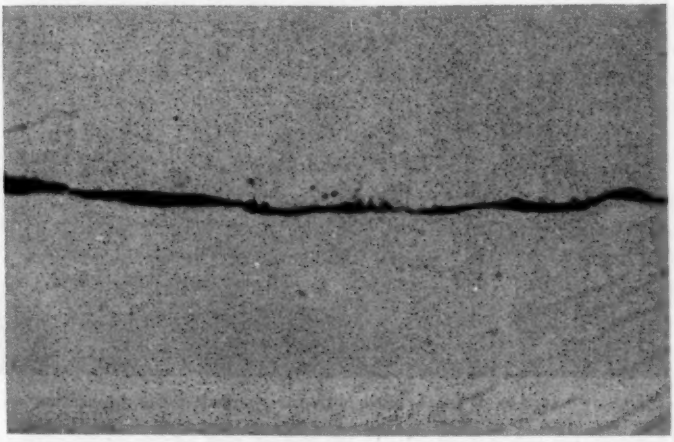

FIG. 2.—Section similar to that shown in Fig. 1. The section has been treated with Schiff's reagent, and the dentin adjacent to the cavity is strongly colored. (Orig. mag. ×450.)

fore, that the dimethacrylate was essential for the production of the altered dentin zone. However, in the test tube no color develops if glycophospheric acid dimethacrylate is added to Schiff reagent; so it appeared that a third factor was necessary.

Methyl methacrylate monomer, with or without hydroquinone stabilizer, does give a direct Schiff reaction in the test tube. It seemed possible, therefore, that the glycerophosphoric acid dimethacrylate, while not itself giving a color reaction with Schiff's reagent, might influence the polymerization of the monomeric methyl methacrylate so that a part of this compound would remain Schiff-reactive. In order to test the hypothesis that a combination of monomeric methyl methacrylate and glycerophosphoric acid dimethacrylate was necessary for the results observed, experiments were performed in which cavities were painted with the dimethacrylate and then filled with silicate cement, which, of course, contains no methyl methacrylate monomer. No Schiff-reactive zone could be demonstrated, thus supporting the hypothesis that methyl methacrylate monomer is necessary for the reaction.

There were also a number of other possibilities to be considered. The self-polymer-

izing resin is activated by paratoluene sulphinic acid, a compound which has been shown to give a slowly developing direct Schiff reaction.[5] To investigate the possibility that the positive reaction in the zone of altered dentin might be due to a "capturing" of paratoluene sulphinic acid by the glycerophosphoric acid dimethacrylate, further cavities were prepared and filled with another resin having a different activator system. The zone of altered dentin, giving both increased affinity for hematoxylin and the direct Schiff reaction, was again present wherever the cavity seal or glycerophosphoric acid dimethacrylate had been applied. As the resin filling material used in this series contained no paratoluene sulphinic acid, the altered staining and Schiff reaction could not be due to this compound.

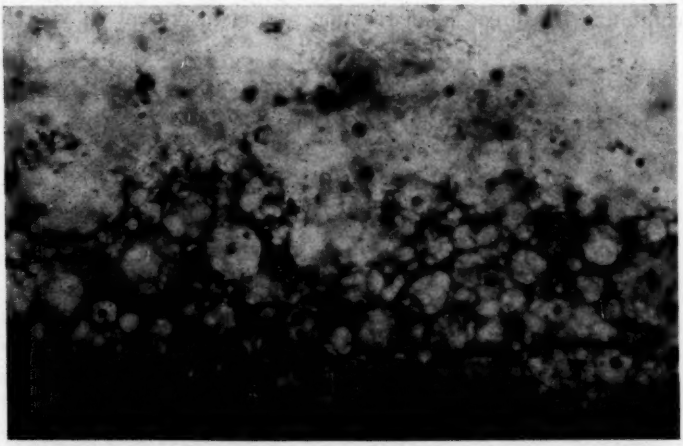

FIG. 3.—Undecalcified section of tooth filled with the resin after the application of glycerophosphoric acid dimethacrylate to the cavity floor. The section was treated with Schiff's reagent, and the material between the polymer granules in the deepest part of the cavity gave a strong color reaction. The filling-dentin junction lies just below the lower border of the photomicrograph. (Orig. mag. ×175.)

The possibility of some interaction between the dimethacrylate and material from the mouth contaminating the cavities during preparation was considered, but identical results were obtained in cavities cut after extraction. The interaction of glycerophosphoric acid dimethacrylate with the formaldehyde of the fixative was an obvious possibility, as Schiff's reagent is used mainly for the detection of aldehyde groups. However, teeth not exposed to any fixative gave the same results. It was also found that the direct Schiff reaction could be obtained from the region of the zone of altered dentin even in undecalcified sections; hence the production of Schiff-positive material by acid hydrolysis during decalcification could be excluded. In those undecalcified sections in which the filling was preserved in the sections, the direct Schiff reaction could also be obtained in the deeper parts of the filling mass. Here the reactive material was lying between the polymer granules, in the areas which had been composed of monomer plus the glycerophosphoric acid dimethacrylate that had become mixed with or dissolved in it (Fig. 3). It was also noted that a band of Schiff-reactive material was present at the junction between filling and enamel; however, it was not possible

to determine the depth of penetration of this material into the enamel substance. Color was present between the prisms, but this might have been due to the diffusion of the colored compound from the enamel surface after reaction with Schiff's reagent, as the filling mass with the strongly Schiff-positive material in the deeper parts was still in position in the sections.

Having thus established that the glycerophosphoric acid dimethacrylate was essential for the altered staining reactions and the Schiff reaction and that material from the mouth, products of acid hydrolysis, aldehyde groups from formaldehyde, and paratoluene sulphinic acid from the overlying resin could all be excluded, we decided to investigate the possibility that the results were due to the interaction of the dimethacrylate and some constituent of the tooth substance either occurring naturally or produced during cavity preparation.

For this purpose, it was necessary to find some material in which cavities could be prepared which was easy to section and which did not normally stain with hematoxylin or react with Schiff's reagent. Polyvinyl chloride (p.v.c.) fulfilled these requirements; hence the various experiments were repeated with cavities cut in the outer aspect of the wall of thick-walled p.v.c. tubing. Negative results were obtained when ordinary histologic sections were prepared, but this procedure involved removal of the filling, and it seemed likely that these negative results were attributable to lack of penetration of the glycerophosphoric acid dimethacrylate into the p.v.c. When slices were prepared, with the filling still *in situ*, positive Schiff reactions were obtained where the filling was in contact with the area to which the dimethacrylate had been applied. Therefore, it could be concluded that the Schiff reaction was not due to a compound formed from the interaction of glycerophosphoric acid dimethacrylate and tooth substance.

When studying the effects of the cavity seal (or a mixture of similar composition) on the dentin by histologic methods, Buonocore and Quigley[3] considered that there might be a reaction between the glycerophosphoric acid dimethacrylate and the organic constituents of the dentin. In the present series of experiments an observation was made that supported this view. It was found that, while teeth fixed in formalin and subsequently exposed to alcohol during dehydration showed both the altered staining and the direct Schiff reaction in the zone of affected dentin, teeth fixed in alcohol showed only the altered staining; the direct Schiff reaction was negative. The Schiff reaction was also negative if alcohol fixation was followed by exposure to formalin.

This suggests that the Schiff-positive material is alcohol-soluble but that it can be rendered resistant to alcohol by exposure to formaldehyde. This might, of course, be due to a direct reaction between the material and the formaldehyde. However, it might also be due to an effect of the formaldehyde on the dentin matrix, with a consequent binding of the Schiff-positive material so that it could no longer be removed with alcohol.

DISCUSSION

Two alternative hypotheses could explain the results obtained. First, the direct Schiff reaction may be the same as that which causes the methyl methacrylate monomer to give the reaction and that the effect of the glycerophosphoric acid dimethacry-

late is to cause a part of the monomer to remain reactive even after the polymerization reaction has occurred. In the absence of glycerophosphoric acid dimethacrylate the mass becomes non-reactive when the monomer is polymerized. Second, the Schiff-positive substance may result simply from some change occurring in the glycerophosphoric acid dimethacrylate during the polymerization process. Whatever the chemical explanation of the direct Schiff reaction, the following points emerge from these investigations:

1. The glycerophosphoric acid dimethacrylate is the component of the cavity seal liquid responsible, directly or indirectly, for the altered staining of the dentin previously reported.[1] The experiments of Buonocore *et al.*[2] indicate that the same component is responsible for the adhesion.

2. Following the application of the cavity seal or glycerophosphoric acid dimethacrylate to the floor of a cavity, the material effects a limited penetration into the dentin matrix; when a self-curing acrylic resin filling is inserted, part of the glycerophosphoric acid dimethacrylate also dissolves in, or mixes with, the monomer of the filling.

3. The distribution of the glycerophosphoric acid dimethacrylate, both as a result of penetration into the underlying dentin and as a result of solution in the monomer of the overlying resin filling, can be demonstrated by the direct Schiff reaction.

4. The material giving the direct Schiff reaction depends on the presence of the glycerophosphoric acid dimethacrylate, although this material itself is Schiff-negative, and results either from altered polymerization of monomeric methyl methacrylate or from a compound formed during the polymerization reaction when glycerophosphoric acid dimethacrylate is present.

It is a pleasure to acknowledge the technical assistance of Mr. A. Smith in this investigation. The glycerophosphoric acid dimethacrylate used in the experiments was kindly supplied by the Amalgamated Dental Company Limited.

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Histochemical Analysis of Lesions in Incipient Dental Caries. II. Sulfhydryl and Disulfide Radicals

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In a previous report[1] a number of histochemical changes preceding cavitation were shown to occur in the dentin just beneath the dentinoenamel junction. These included a cessation of oxidation reduction, an increased stainability of carbohydrate and protein, the presence of a silver nitrate–reducing agent, and the loss of some mineral. The present paper extends these observations to a consideration of the possible presence of sulfhydryl and disulfide radicals in the dentinal tubules under normal and pathologic conditions.

The number of histochemical techniques which may be applied to sections as prepared[1] is limited. Those reactions which require an acid medium, in general, will not work because the tooth structure cannot be kept at an acid pH until decalcification is almost complete. Unstable compounds may not withstand such treatment. Furthermore, the action of acid upon the structure under study may alter the structure to such an extent as to make valid conclusions impossible.

MATERIALS AND METHODS

One hundred Osborne-Mendel caries-susceptible rats were utilized in this study. The animals were divided into two groups at 20 days of age. One group was placed on the cariogenic diet previously outlined,[1] while the other group was placed on a noncariogenic diet.* Littermates from the two groups were sacrificed at various times between 20 and 30 days of age, and the jaws were frozen and sectioned by the method developed in this laboratory.[1]

The ditetrazolium chloride method[2] may be used for the demonstration of sulfhydril and disulfide groups, but in tooth sections the color is weak and the definition poor.

Excellent definition may be obtained in tooth sections for the sulfhydryl and disulfide groups by the lead acetate method.[3] It is well known that glutathione, if heated with an alkaline solution of lead acetate, is converted to lead sulfide.[4] Sulfhydryl and disulfide groups within the dentinal tubules were visualized by the following method. The prepared sections were placed for 2–6 hours in a solution of lead acetate containing lead acetate, 1.0 gm.; sodium hydroxide, 0.1; and distilled water, 25 ml. The sections were washed quickly in 0.2 per cent nitric acid, washed four times in 50 per cent alcohol, dehydrated with ethyl alcohol, and mounted.†

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* Purina Laboratory chow, Ralston Purina Co., St. Louis 2, Mo.

† Permount, Fisher Scientific, New York.

RESULTS

The black precipitate, presumably lead sulfide, is shown in the dentinal tubules in Figure 1. Sections treated with 3 per cent hydrogen peroxide for 2 hours to oxidize the sulfhydryl groups did not develop a black precipitate with lead acetate (Fig. 2). Sections initially treated with a 1 per cent aqueous solution of N-ethyl-maleimide, widely used as a specific sulfhydryl inhibitor, did not develop a black precipitate within the tubules when treated with the lead acetate solution (Fig. 3). If the sections were first treated with lead acetate and then exposed to hydrogen peroxide, a yellow-brown coloration, typical of the action of peroxide on lead sulfide, was produced. Thus it would appear that the black precipitate found in the tubules may be lead sulfide formed

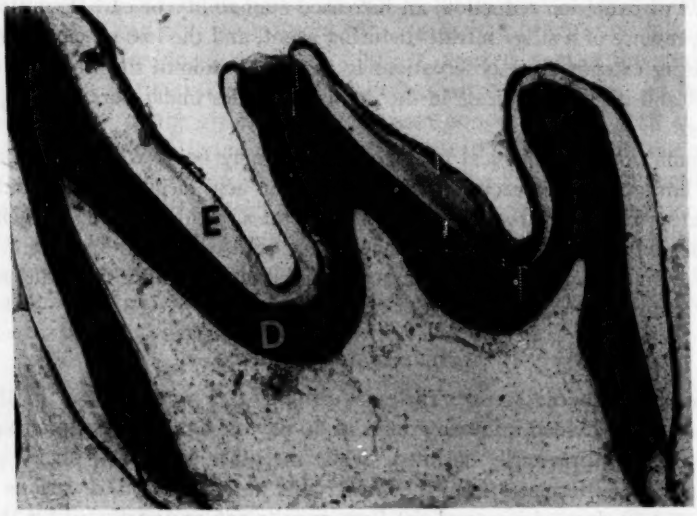

FIG. 1.—Section treated with lead acetate. Note black precipitate in tubules. D = dentin; E = enamel.

from the interaction of the lead acetate with the sulfhydryl of the dentinal tubules.

A typical section from an animal on the non-cariogenic diet until 30 days of age treated with the lead acetate is shown in Figure 4. A typical section from a 30-day-old littermate fed on the cariogenic diet and treated with lead acetate is shown in Figure 5. The area of pathosis is devoid of the tubular precipitate. This may be found as early as 26 days of age in animals on the cariogenic diet. The sharp line of demarcation between the affected and the unaffected dentin is usually present at 28 days of age.

DISCUSSION

It is well known that sulfhydryl groups are essential in a number of enzyme systems.[5] It was previously shown that there occurs a failure of the oxidation-reduction system within the area of early pathology.[1] The evident diminution of reactive sulfhydryl groups in the incipient carious lesion extends the description of this phenomenon and further implicates the organic phase of the tooth in the pathogenesis of this dis-

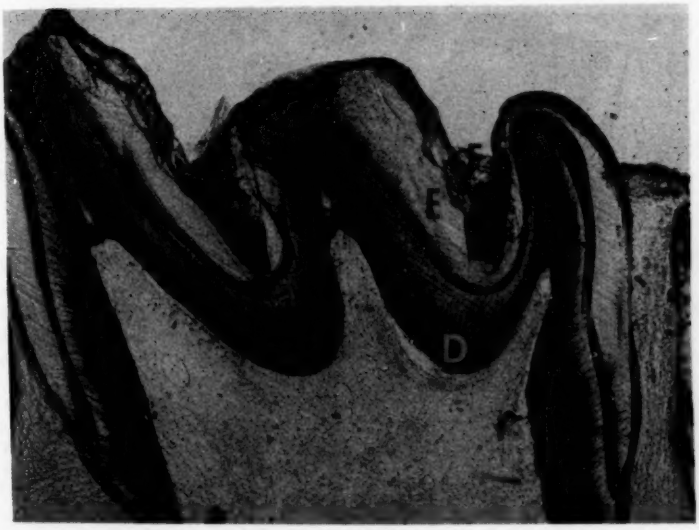

FIG. 2.—Section treated with 3 per cent hydrogen peroxide before treating with lead acetate. *D* = dentin; *E* = enamel; *F* = food.

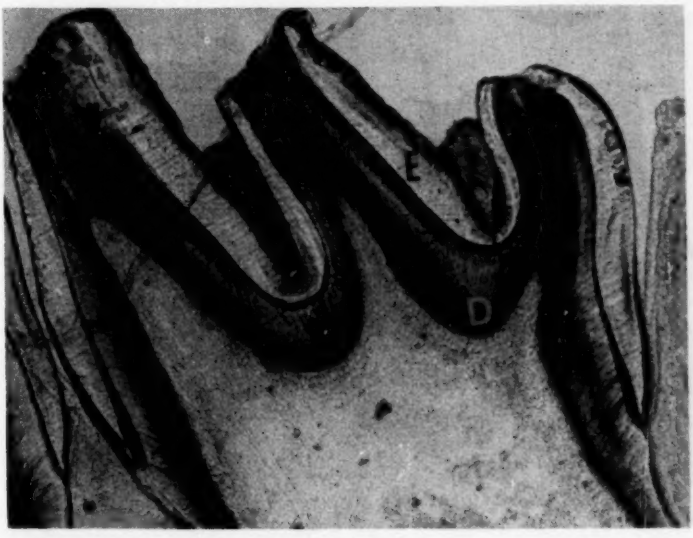

FIG. 3.—Section treated with N-ethyl-maleimide before treatment with lead acetate. *D* = dentin; *E* = enamel; *F* = food.

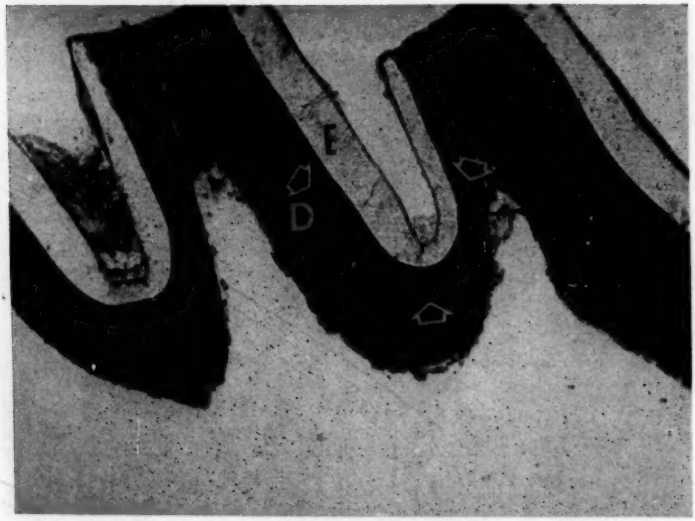

FIG. 4.—Section of animal on non-cariogenic diet until 30 days of age treated with lead acetate. Note black precipitate in tubules. *D* = dentin; *E* = enamel; *F* = food.

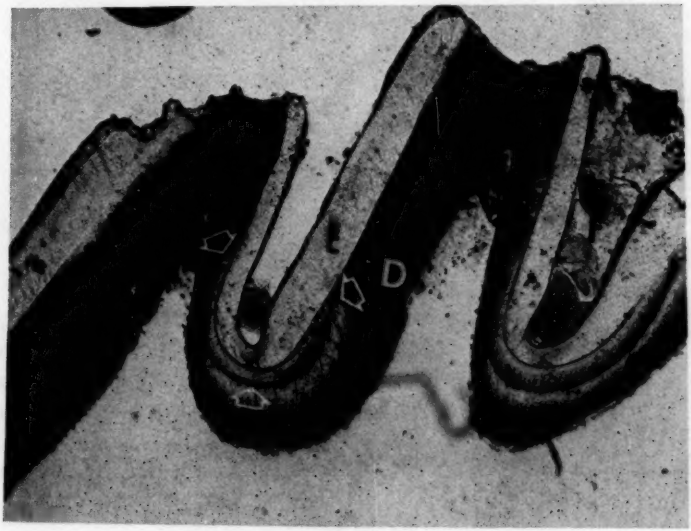

FIG. 5.—Section of animal on cariogenic diet until 30 days of age treated with lead acetate. Note area of early pathology. *D* = dentin; *E* = enamel; *F* = food.

ease. Further studies are being conducted on the chemical identity of the affected radicals and on the microbial agencies which apparently block them.

SUMMARY

A sensitive method for the possible demonstration of sulfhydryl radicals within the dentinal tubules was presented. Evidence that sulfhydryl groups disappear during the pathogenesis of incipient dentinal caries was shown.

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Dental Anatomy and Characteristics of Periodontal Lesions in the Mongolian Gerbil

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A number of animal species has been employed in the experimental production of various types of periodontal lesions.[1-12] The rice rat[11, 12] is an excellent animal for this type of investigation; likewise, the hamster has been used frequently.[8, 9] During a preliminary survey of eight rodent species which was undertaken to find an animal

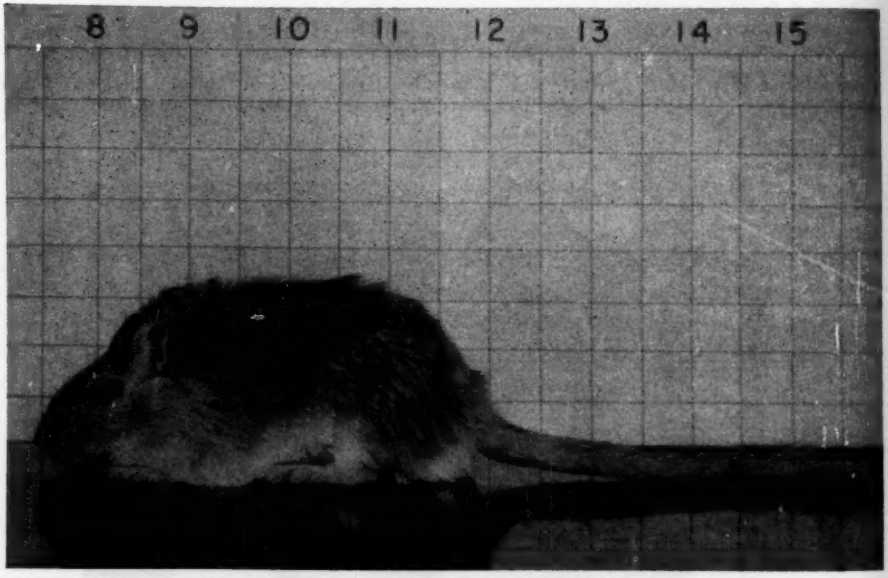

FIG. 1.—Mongolian gerbil. The scale in the background is in inches

that was highly susceptible to periodontal disease, each of the six available representatives of the Mongolian gerbil had a low incidence of small lesions in the oral soft and calcified tissues, indicative of an inherent susceptibility in this species. The present study was undertaken to determine whether the Mongolian gerbil would be suitable for use in the laboratory for the study of periodontal disease.

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The Mongolian gerbil (*Meriones unguiculatus*) (Fig. 1) belongs to one of the great divisions of ancient mice. This division consists of about a dozen genera. They are all small, ranging in size from that of mice to that of rats. They are root and seed eaters and burrow industriously under grass and scrub.[13]

The Mongolian gerbil is widely distributed throughout North Africa, southeastern Russia, northeastern China, and eastern Mongolia.[14] It was introduced into the United States by way of Japan in 1954. It is polyestrous, breeding throughout the year, and has a gestation period of 23–24 days. The maximum litter size is said to be 8, and the usual number is 4 or 5.[15] This gerbil is an almost ideal laboratory animal because it exhibits a broad spectrum of susceptibilities to disease[16] and is readily amenable to handling and to the circumstances associated with captivity in the laboratory.

EXPERIMENTAL METHODS

In a preliminary study, 14 young adult Mongolian gerbils between the ages of 12 and 30 weeks were sacrificed for the study of the periodontium at the time that they were received from the animal dealer without any experimental preparation.

In a second study, 42 weanling Mongolian gerbils were divided into four groups of 11, 10, 10, and 11, respectively. Nine gerbils died in the early weeks of the experiment, leaving groups of 8, 9, 10, and 6, respectively, that were suitable for experimental purposes. The distribution of males and females, as well as littermates, in each group was kept as uniform as possible. The animals in the first group were maintained on Harvard ration #700,[17] which is purified, finely powdered, and cariogenic. The subjects in the second group were fed Keyes ration #4,[18] which is finely powdered and also cariogenic. The gerbils of the third group were given Purina laboratory chow, and those in the last group received a mixed-grain ration consisting of cracked corn, wheat, oats, barley, buckwheat, and a pelleted protein supplement that was composed of soybean oil meal, linseed oil meal, and powdered skim milk. The average protein content of this diet was 24 per cent. All the animals were housed in individual wire-bottom cages and were given their respective diets and water ad libitum.

In order to appraise the extent and severity of periodontal lesions, about one-sixth of the gerbils in each group were killed at ages 17, 23, 26, and 28 weeks and the remaining third at 43 weeks of age. The heads were kept in 95 per cent alcohol for 48 hours and then skinned. The periodontal tissues were examined under a binocular microscope at a magnification of 30×.

The method used in the evaluation of the extent of periodontal lesions in the Mongolian gerbil was essentially the same as previously described for the rice rat.[12] Only those modifications were made which were necessary to adapt to differences in the tooth anatomy between the two species. The deep grooves on the lingual and buccal surfaces of molars of the gerbil divide each surface into several prominences which can be conveniently used to define areas affected by lesions of the periodontium. The first molar has two grooves and three prominences on each of the lingual and buccal surfaces, while the second molar has one groove and two prominences. The third molar has only one prominence. These prominences were taken as the reference points. Thus there were 6 areas on the lingual and 6 on the buccal surfaces of the teeth in each quadrant, making a total of 48 areas, in contrast to the 56 comparable areas in the

rice rat. The lesions around these 48 areas were scored with values ranging from 1+ to 4+ with the individual criteria described previously.[12]

RESULTS

The gerbils of the first study were used to examine the dental anatomy of the species in detail. The dentition of the gerbil is monophyodont, polybunodont, and brachyodont, with the typical rodent dental formula:

$$I\ \tfrac{1}{1},\ C\ \tfrac{0}{0},\ Pm\ \tfrac{0}{0},\ M\ \tfrac{3}{3}.$$

Dental anatomy.—The incisor teeth show characteristics similar to those of other rodents. They are of continuous eruption throughout life and are separated from the molar teeth by a large diastema.

Photographs of the occlusal, buccal, and lingual or palatal surfaces of molars are shown in Figure 2. The molars in each jaw are arranged in a straight line. The maxillary and mandibular molars are strikingly similar. The size decreases strikingly from the first to the third molar. The third is very small in comparison with the first or the second. The crowns of the molars are, on the average, about one and one-half times the length of the roots and are longer than those of the rice rats and hamsters. The buccal and lingual surfaces of the first and second molars have grooves of various depths. These grooves reach from the cervix to the occlusal surface and partially divide the crowns into several lobes. The third molar has a cylindrical crown without grooves on lingual or buccal surfaces. The occlusal surfaces of all molars are almost flat and without cusps or fissures. There is a thin, raised enamel margin on the periphery of the occlusal surface, but the rest of this surface has no enamel covering. The cemento-enamel junction is not clearly demarcated.

The first molars are the largest in their respective arches. Each of the buccal and lingual surfaces of the crown has two deep grooves which partially divide the occlusal surface into three lobes. Each of the first molars has three roots: mesial, distal, and lingual. The mesial roots are thick, rounded, and bulbous at the apex; the root length is but slightly less than the crown length. The distal roots are shorter and thinner than the mesial, the root length is approximately one-third the crown length, and the apex is slightly thickened. The lingual root is the shortest and is straight and very thin. It is slightly longer than the distal in the maxillary molars, while it is shorter than the distal root in the mandibular molars.

The maxillary and mandibular second molars are about two-thirds the size of the first. There is one deep groove on the buccal surface and one on the lingual surface of the crown, thus partially dividing the occlusal surface into two lobes. These have two roots: mesial and distal. The mesial is short, thin, and slightly curved toward the distal side; the root length is about one-third the crown length. The distal root is longer and thicker than the mesial; the apical part is slightly thicker.

The maxillary and mandibular third molars are the smallest of the molars. They are about one-third as large as the second molars and are not subdivided into lobes. The crown is round and cylindrical in shape. The third molars have one cylindrical root, which is slightly longer than the crown. The root leans mesially, with its bulbous apex toward the distal side.

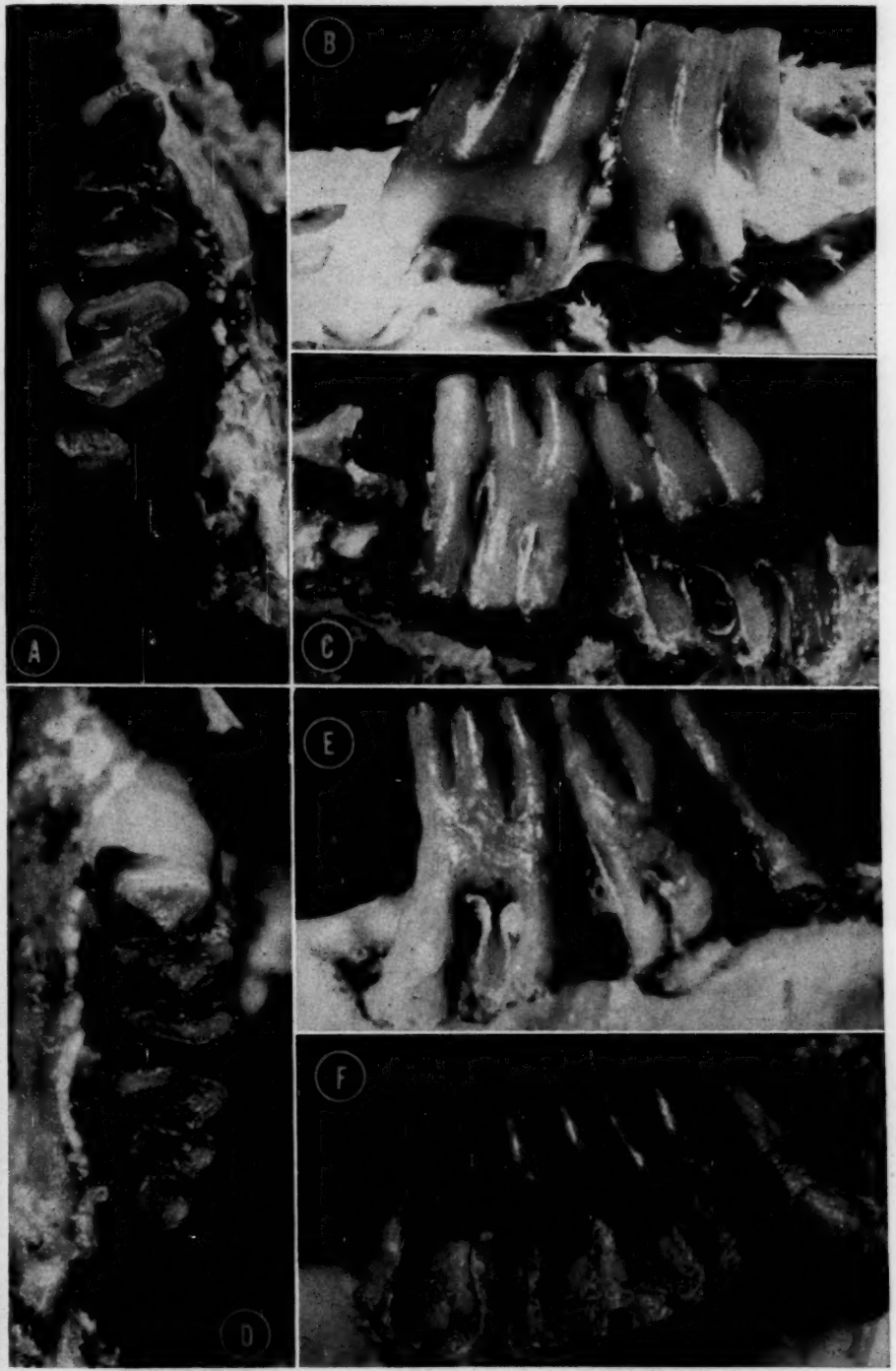

FIG. 2.—Photographs of fixed preparations of the jaws of the Mongolian gerbil. (Orig. mag. ×15.) *A*, Occlusal view of right maxillary molars. *B*, Buccal view of right maxillary molars. *C*, Lingual view of right maxillary molars. *D*, Occlusal view of right mandibular molars. *E*, Buccal view of left mandibular molars. *F*, Lingual view of right mandibular molars.

Dietary evaluation.—All the gerbils in the second study adapted themselves readily to the laboratory conditions. During the 40 weeks of the experimental period, there was no evidence of susceptibility to intercurrent infection. Laboratory handling was facilitated by the docile temperament of these animals.

All showed a steady weight gain. The males were consistently heavier than the females. The average weight of the adult male gerbils at 20 weeks of age was approximately 95 gm., and that of females of the same age was about 85 gm. Those fed ration #700 were slightly heavier than those given any of the other diets. The coats of the animals on all four diets were smooth and shiny. The animals showed no indication of nutritional deficiencies.

Lesions of the soft and calcified tissues of the periodontium occurred in almost every animal. The severity of the lesions was dependent on the length of experiment. Lesions were minor up to about 25 weeks. Slight to moderate lesions were observed at about 35 weeks.

FIG. 3.—Maxillary molars. Note impaction of hair and food debris in the lingual pockets, an early stage of periodontal disease.

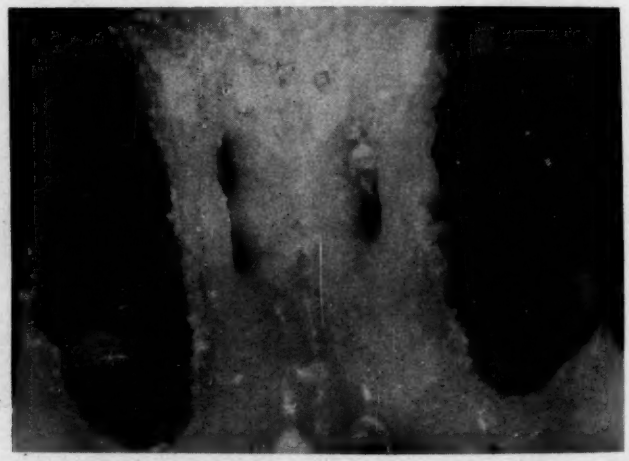

FIG. 4.—Specimen of Fig. 3 after the removal of soft tissues. Note the areas of slight bone resorption

The lesions in the soft tissues showed characteristics which varied with the different diets. The soft-tissue lesions of those animals fed the mixed-grain diet were characterized by discrete areas of hair and food impaction. The impactions were located at the interdental areas and between the dental grooves and the gingival tissue. The gingival tissue surrounding the impactions was normal in color and consistency. The interdental papillae were knife-edged and appeared normal (Fig. 3). After removal of the soft tissues, areas of bone resorption were observed in the same areas as the hair and food impactions (Fig. 4).

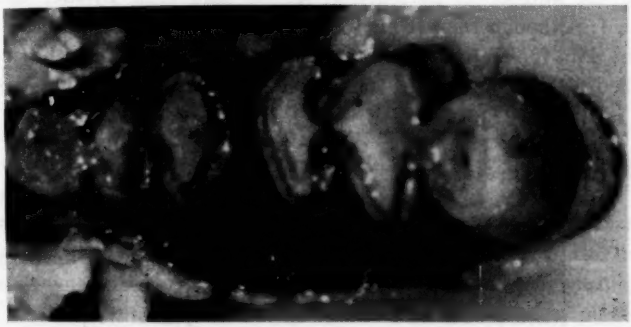

FIG. 5.—Right maxillary molars. Note relatively advanced periodontal disease, with heavy impaction of food debris and the detachment of gingival tissue.

FIG. 6.—Specimen in Fig. 5 after the removal of soft tissues. Note the advanced bone resorption

The soft-tissue lesions in animals maintained on laboratory chow were similar to those on mixed grain but were not so severe. The hair in the impacted areas was scantier, and there was more food debris. The impactions were largely interdental and were less numerous in the dental grooves and the gingival tissue. The calcified-tissue lesions were generalized and showed greater bone loss at the site of impaction.

The soft-tissue lesions of animals maintained on rations #700 and #4 were similar to each other in characteristics and were more generalized in nature. The gingiva was generally edematous. Recession and detachment of the gingival tissue were very prominent; the interdental papillae were flabby and edematous. No solitary discrete impactions were observed, and no hair was lodged in the gingival tissue. Bone loss was generalized (Figs. 5 and 6).

Periodontal lesions were more frequent on the lingual than on the buccal side. The maxilla was more severely affected than the mandible. A high degree of bilateral symmetry of the lesions was observed. Those teeth with relatively extensive lesions were slightly mobile. A calculus-like material occurred frequently on the mesial surfaces of the maxillary first molars. A few teeth of animals maintained on rations #700 and #4 were carious.

The frequencies of soft- and calcified-tissue lesions in the animals maintained on the four diets were evaluated in terms of the number of molars with contiguous periodontal lesions, the number of periodontal lesions, and their extent; these data are given in Table 1. Although the gerbils were killed at five different times, it is reasonable to pool the data for each ration because a similar proportion of the gerbils in each group was killed at each interval. From Table 1 it can be seen that diets of mixed

TABLE 1

EFFECTS OF DIFFERENT RATIONS ON PRODUCTION OF PERIODONTAL LESIONS IN MONGOLIAN GERBIL*

Group	Ration	No. of Animals	Soft-Tissue Lesions			Calcified-Tissue Lesions		
			No. of Molars with Contiguous Periodontal Lesions Av.†	No. of Periodontal Lesions Av.†	Extent of Periodontal Lesions Av.†	No. of Molars with Contiguous Periodontal Lesions Av.†	No. of Periodontal Lesions Av.†	Extent of Periodontal Lesions Av.†
1.....	700	8	6.0	21.1	29.1+	3.0	6.0	6.3+
2.....	4	9	5.5	16.5	20.3+	2.9	6.8	6.8+
3.....	Laboratory chow	10	5.9	19.4	27.1+	4.4	10.8	13.2+
4.....	Mixed grain	6	5.6	19.6	31.6+	5.2	15.0	18.0+

* Animals sacrificed between 17 and 43 weeks of age.

† Average value for one-half of both jaws of all animals in each group.

grain, laboratory chow, and ration #700 did not cause greatly different incidences of soft-tissue lesions when expressed by the scoring system used. The animals maintained on the mixed-grain diet, laboratory chow, and ration #700 had an almost identical amount of soft-tissue damage. Animals fed finely powdered ration #4 had the lowest incidence of soft-tissue lesions. Animals on the mixed-grain diet had the greatest number and extent of calcified-tissue lesions. Those on laboratory chow had the next most severe calcified-tissue lesions. However, animals on rations #700 and #4 had the lowest incidence of calcified-tissue lesions. These data indicate that modest variations in the incidence of the periodontal syndrome occurred in this animal during maintenance on various common laboratory diets. However, the importance of these differences cannot be evaluated at the present time because of the small groups and the successive time intervals at which they were sacrificed, which makes any statistical evaluation impossible.

From the periodontal studies of the rice rat and of hamsters it has been observed that the destruction of calcified tissues is more severe in proportion to soft-tissue destruction.[19–21] However, in the gerbil used in this instance the calcified-tissue lesions were less severe than the soft-tissue lesions.

DISCUSSION

From the results of the present study, it is evident that the Mongolian gerbil can be easily maintained in the laboratory environment on common laboratory diets that are suitable for studies on the production of the periodontal syndrome. The periodontal lesions as observed in this species were very mild, even on diet #4, which has previously been shown to produce severe periodontal lesions in hamsters and the rice rat under comparable laboratory conditions.[8, 9, 19, 20] Although the lesions in animals on rations #700 and #4 appeared to have characteristics similar to those in humans, there were several disadvantages in the routine use of this animal for periodontal studies. In order to develop periodontal lesions of sufficient severity, periods as long as 40 weeks were required. In addition, the mild character of periodontal lesions might present a great problem in situations where slight differences in lesions were encountered. The mild susceptibility to periodontal disease in this species at present can possibly be overcome by careful breeding and selection procedures, provided that all other considerations are favorable.

The calcified-tissue lesions in this animal were difficult to evaluate accurately because of problems in recognizing the cemento-enamel junction as the line of demarcation of the crown from the root. The crowns of the molars of gerbils are relatively large, with two-thirds of the buccal and lingual surfaces covered by gingival tissues, and these surfaces are deeply grooved. The presence of deep grooves creates a situation that is not comparable with that in the human. The grooves enhance the impaction of food in the adjacent gingiva. The latter anatomical characteristics and the nature of the lesions appear to make this animal less suitable for use in periodontal studies than either the rice rat or the hamster. Therefore, we have not undertaken further studies with the gerbil.

On the basis of current knowledge, the Syrian hamster and the rice rat appear to be decidedly better experimental animals for the production of a periodontal syndrome than the Mongolian gerbil because of the greater similarity between their dental anatomy and that of the human and because of the relatively greater severity of the lesions. However, the major problem with the hamster is its high susceptibility to intercurrent infection, which often destroys entire colonies within a short period. Since the rice rat did not show any evidence of major susceptibility to intercurrent infection and since it has other required characteristics, further experiments to determine its suitability for periodontal research are in order.

SUMMARY

Forty-seven Mongolian gerbils were used to test the adaptability of this species to laboratory circumstances and to study the gross characteristics of its periodontal lesions. The dental anatomy of the Mongolian gerbil is described.

Thirty-three of the gerbils were fed four common laboratory diets. They adapted fully to the laboratory environment and did not show any evidence of unusual susceptibility to intercurrent infection.

Mild periodontal lesions with slight variations in severity were observed on various diets. The mixed-grain diet produced the greatest number and extent of soft- and calcified-tissue lesions.

These animals were not so suitable for laboratory studies of periodontal disease as

were the rice rat and the hamster because of the differences between their tooth anatomy and that of humans and the mild character of the periodontal lesions.

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Determining the Tumorigenicity of Dental Materials

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It has been known for more than a hundred years that certain arsenicals can induce cancer. Literally hundreds of carcinogens have since been recognized. In the early 1930's chromates and nickel carbonyl in metal refineries and asbestos were indicted as carcinogens, prompting a surge of interest in industrial medicine. In recent years, experimental studies involving connective tissue implants of various solids, e.g., films, foils, or powders, and injected liquids, and inhalation tests of many materials have resulted in the development of malignant neoplasms in a variety of animals, including mice, rats, rabbits, dogs, and guinea pigs.[1] Since 1942 the following metals have been used to induce experimental sarcomas: beryllium, selenium, chromium, cobalt, nickel, gold, silver, platinum, and alloys such as vitallium, tantalum, and stainless steel. Most of the tumors developing around such implants, after many months, have been fibrosarcomas; but other forms such as rhabdomyo-, osteo-, lipo-, and reticulum-cell sarcomas have also occurred, sometimes depending on the anatomical site of the implant.[2] Likewise, since 1948, more than a dozen different plastic materials have been shown to induce sarcoma when implanted in rats or mice.[3]

In 1954 a study of methyl methacrylate (acrylic resin) demonstrated that small disks of this dental material implanted subdermally in mice resulted in the formation of sarcomas in 25 per cent of the animals which survived for 469 days.[4] One might gain the impression that any material implanted in rodents would cause sarcomas after long periods of time; however, the following materials have been so tested without demonstrating a tumorigenic effect: fiberglass, cotton, paraffin, wood, mica, glass, fetal fingernails (keratin), and a number of metal foils.[3]

The clinical practice of imbedding metals as substitutes for lost bone due to trauma or surgical excision was not used on a large scale until the years of World War I. Most metallic and plastic implants of this nature have been placed more recently, during and after World War II. Little is known regarding tumorigenesis associated with such implants in human beings.[5] Because of the increasing popularity of implantation techniques in dentistry (e.g., implant dentures, magnets, materials implanted for fracture fixation, metals placed in root apexes after endodontic procedures), the possible tumorigenic qualities of these materials after long periods of time must be considered.

MATERIALS AND METHODS

Since it was known that nickel caused sarcomas in rats,[1] this material was chosen as a positive "control." Since gallium has been suggested as a substitute for mercury in a

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filling material, a nickel-gallium alloy was chosen for study.[6] Ten other materials used in dentistry were chosen rather arbitrarily. Some of these have been classified as carcinogens by others in the past, and others have not been studied in this regard. Pellets (approximately 2 × 2 mm.) of each of the following materials were implanted: fused porcelain beads;* a silicate cement;† an autopolymerizing resin filling material;‡ a denture resin;‡ zinc oxide§ and eugenol;|| calcium hydroxide# and methyl cellulose;|| silver wire;** silver amalgam,†† copper,‡‡ nickel,‡‡ a nickel (60 per cent)-gallium (40 per cent) alloy obtained from the Bureau of Standards; and vitallium, a chrome-cobalt-nickel alloy.

One hundred and twenty Wistar rats, 4–6 weeks of age, were distributed equally by sex into twelve groups. Each animal in each group received four subdermal implants of the same material—two on the dorsal and two on the ventral surfaces. Surviving animals were maintained under comparable, standard laboratory conditions for 27 months. They were observed and palpated periodically to detect any evidence of tissue proliferation at the implant sites. When an animal died or was sacrificed after the first study year, the presence or absence of pellets was determined grossly or by dissection. Regrettably, this procedure was not followed during the first year of the study. All tumors were excised and prepared for microscopic study and occasionally for other study purposes.

RESULTS

Among the ten nickel-implanted animals, five (three males, two females) developed a sarcoma around one pellet. These were detected after 7, 8, 15, 17, and 23 months. Four other animals died during the experiment after 7, 7, 9, and 9 months, respectively, without tumors, and no effort was made to recover pellets from these animals. One was sacrificed after 27 months without tumor, and no pellets were found in this animal.

Nine out of ten nickel-gallium-alloy–implanted animals developed sarcomas during the experimental months numbering 7, 8, 8, 8, 10, 11, 12, 13, and 13. One animal died after 11 months without tumor, and pellets were not sought in this animal. One animal developed two sarcomas, both apparent by 9 months, which increased in size for 3 months until one was 6 × 10 × 4 cm. and the other was 2 × 2 cm. A sarcoma which developed after 8 months in one animal was excised, and some finely divided tumor tissue was reimplanted at a new site in the same animal. New growth soon was apparent, which was observed to increase for 1 month until sacrifice and microscopic confirmation of the diagnosis. Other attempts were made to transplant the same tumor to other animals without success. After two fruitless attempts, another sarcoma was

* Fused feldspathic porcelain pellets generously prepared by the Dentist's Supply Co., New York.

† S. S. White Mfg. Co., Philadelphia.

‡ Kadon (autopolymerizing resin) and Lucitone (a processed denture resin), L. D. Caulk Co., Milford, Del.

§ USP Merck & Co., Rahway, N.J.

|| Indiana University Pharmacy, Indianapolis. Ind.

J. T. Baker Chemical Co., Phillipsburg, N.J.

** Indiana University Medical Center, Indianapolis, Ind.

†† Mynol, Alloy Improved, Mynol Chemical Co., Philadelphia.

‡‡ Indiana State Board of Health, Indianapolis, Ind.

transplanted successfully in vitro, and growth in tissue culture was observed for several months.

In general, the tumors developing at the subdermal implant sites in these two groups of animals grew rapidly, often ulcerated, sometimes metastasized widely, and at times caused death before the animals were sacrificed (Fig. 1). For example, one tumor was detected when it was 1 × 1 × 1 cm. in size, and 4 days later it had increased to 7.5 × 5 × 3 cm. A previous investigator, using powdered nickel, described tumors which he detected when they were "golf-ball size" and which grew to "tennis-ball size" in a matter of days.[1]

FIG. 1.—A fibrosarcoma occurring around a nickel pellet after 8 months of implant time

The cytology of the lesions (Figs. 2, 3, and 4) ranged from fibrous "spindle" cells, through "round" cells to "giant cells" in different tumors, and sometimes in the same lesion. Occasionally, large eosinophilic, multinucleated masses resembling giant cells were seen to be striated, resembling rhabdomyosarcoma cells.

No animals of any other group developed tumors related to the implants. From four to eight animals of each of the ten groups other than nickel and nickel-gallium survived more than 20 months, and representatives of all groups survived until sacrifice after 27 months. Pellets were recovered from some animals of every group but copper, and sections were made through the uniformly thin, non-inflammatory, acellular, fibrous capsules around them. The survival of the animals in the copper group, in months, was 2, 7, 13, 18, 21, 24, 24, 24, 24, and 27. However, no pellets were detected

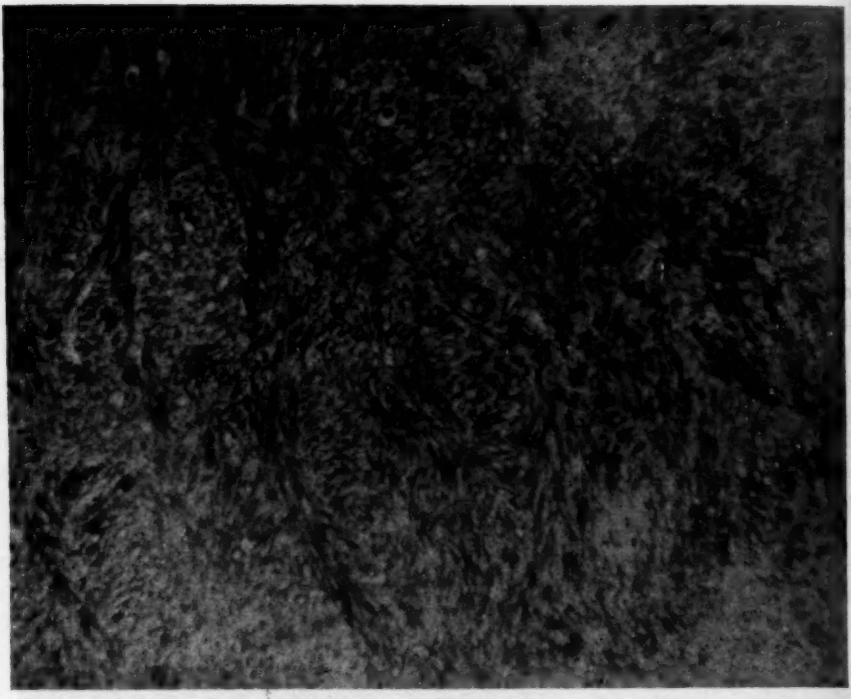

FIG. 2.—Purposeless whorls of spindle-shaped fibroblasts from one part of a sarcoma around a pellet of nickel-gallium alloy.

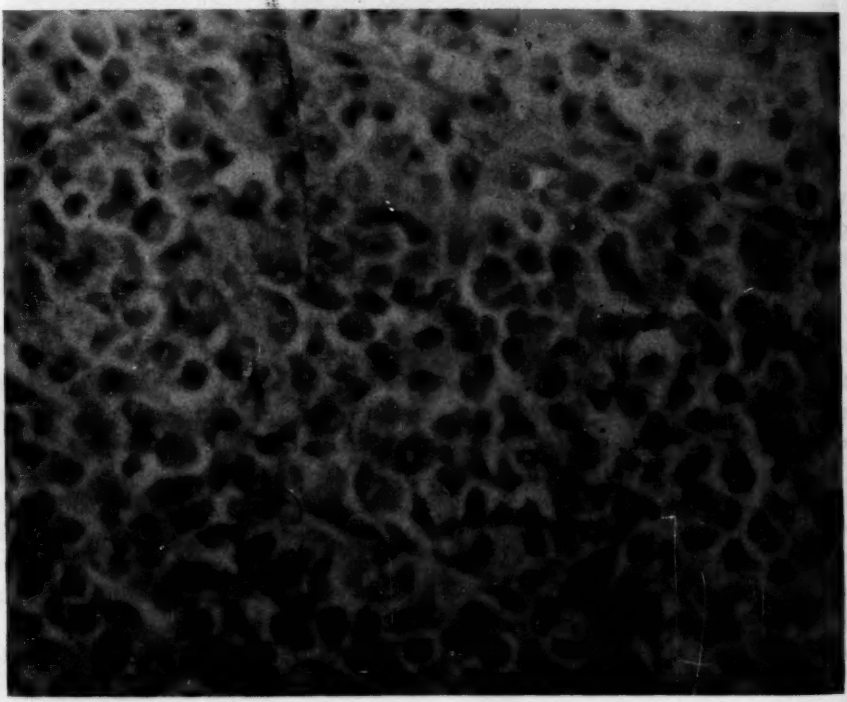

FIG. 3.—A high-power view of round cells from a part of a sarcoma around a nickel pellet. Note the mitotic figures, pleomorphism, and variations in staining intensity.

in any of these animals after death. Since studies have shown that copper is a strong irritant,[8] it is possible that these pellets sloughed soon after implantation, and thus a true measure of the tumorigenicity of this material may not have been obtained.

Several fibroadenomas of the mammary glands developed[7] in the older animals, but these were not found to be in close relationship with the various pellets implanted in these animals.

FIG. 4.—Photomicrograph of the same tumor seen in Fig. 2, showing the skin and its appendages over the tumor, which is made up of fibrous tissue and large multinucleated cells. Some of the latter occasionally resemble muscle fibers. The implants were placed in the region of the panniculus carnosus.

DISCUSSION

In future studies, more careful and more frequent observations of the animals should be made during the early weeks after implantation to see that the materials are retained. Also, at the time of death, the presence or absence of materials should be ascertained in every instance by dissection or possibly through the use of radiographs. Unfortunately, such careful measures were not taken in this study until after several animals had died during the first year.

No real differences in the tumorigenicity of nickel and the nickel-gallium alloy were demonstrated, because of the early deaths of four non-tumor-bearing animals of the nickel group. It is not known whether gallium has any tumor-inducing qualities per se, although it may be said conclusively that nickel alone or in combination with gallium appears to be very tumorigenic. Gallium, along with other materials, is being studied further.

Why vitallium, silver, denture resin, and the autopolymerizing resin did not induce tumors, as might have been expected from earlier studies in rodents, is not known. Perhaps the physical form of the small pellets may be a factor, since other implants have been foils, disks, and powders, but chemical properties and reactions of such materials from different sources must also be considered.[9] Nonetheless, it is felt that the *relative* tumorigenicity of these several materials in a solid pellet form has been well demonstrated in rats. The significance to mankind of these findings in animals is, of course, not clear. The experiment with human beings is being conducted now, in clinical practices over the world, and many careful case histories must be collected over many years[5] in order to evaluate the results.

SUMMARY

One hundred and twenty young adult Wistar rats were divided equally by sex into twelve groups. Four subdermal implants of small pellets of twelve materials were placed in each animal and observed for up to 27 months. Five of ten animals developed a sarcoma around a nickel pellet, and nine of ten animals developed one or more sarcomas around pellets of a nickel-gallium alloy. No tumors occurred around fused porcelain, a silicate cement, an autopolymerizing resin, a denture resin, zinc oxide and eugenol, calcium hydroxide and methyl cellulose, silver wire, silver amalgam, vitallium, and copper. The validity of the results obtained in the copper group may be questioned, but it appears that this is a satisfactory method for study of the relative tumorigenicity of many dental materials prepared in various physical forms.

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Bacteriophage Typing and Antibiotic Sensitivity Testing of Staphylococci Isolated from the Oral Cavity of Man

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Staphylococci are ubiquitous and currently constitute a serious problem in the field of infectious diseases. Considerable data are available pertaining to the incidence, biochemical properties, and cultural characteristics of the various species of micrococci which may be found in the human mouth.[1–6] Except for the work of Taplin and Goldsworthy,[7] we have, thus far, not encountered additional studies in the dental literature whereby investigators have used bacteriophages in identifying coagulase-positive staphylococci isolated from the oral cavity or from teeth.

The identification and differentiation of a culture of *Staphylococcus aureus* has been aided and simplified by bacteriophage typing.[8] In the present study we have utilized this technique and wish to report both the bacteriophage patterns and the susceptibility to penicillin,* oxytetracycline,* and oleandomycin,* of a number of coagulase-positive strains which have been isolated from the oral cavity of man.

MATERIALS AND METHODS

A total of two hundred carious teeth, each from an individual patient, were extracted and planted† directly into 20 × 150-mm. screw-cap test tubes containing Brewer-modified thioglycollate broth.‡ It was decided to use teeth as the source of mouth bacteria because of the simplicity and ease of handling of this material. The inoculated broth was first incubated for 2–3 days at 37° C. and then streaked to Staphylococcus agar 110.§ On this medium most coagulase-positive staphylococci will appear as brown-yellow colonies after 24–72 hours of incubation. Coagulase-negative staphylococci and other species of bacteria usually produce non-pigmented colonies or will not grow. This was confirmed by coagulase tests, using fresh human plasma as the substrate.

Phage typing was accomplished with an apparatus|| whereby the following staphylococcal phages were deposited simultaneously on to the surface of an agar plate prior

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* Penicillin, Terramycin, and Matromycin, respectively, manufactured by Chas. Pfizer and Co., Brooklyn, N.Y.

† Collected by Drs. Harvey Salb and Morris Boyer, Union City, N.J., the oral surgeons associated with this study.

‡ Adjusted to contain 70 gm. of sodium chloride per liter. Baltimore Biological Laboratories, Inc.

§ 2.5 ml. of a 1 per cent solution of brom-cresol-purple indicator was added to each liter of medium. Baltimore Biological Laboratories, Inc.

|| U.S. Pat. Pending No. 773057, developed by S. Goldberg, Phage Typing Laboratory, New York City Department of Health.

to seeding the same plate with the organism to be tested: Group I—phages 29, 52, 52A, 79, 80; Group II—phages 3A, 3B, 3C, 55, 71; Group III—phages 6, 7, 42E, 47, 47C, 53, 54, 73, 75, 77, 83; Group IV—phage 42D; 81 and 187 are unclassified.

Each strain was first typed with the routine test dilution (10^{-3}–10^{-5}) of each phage. Strains resistant to lysis by all the phages used in these dilutions were then retyped with concentrated phage (10^{-1} dilution of each phage).

Sensitivity determinations were carried out by means of a broth dilution method.[9] A strain was considered to be susceptible to an antibiotic if its growth was inhibited by 12.5 μg. (or units) or less of the antibiotic per milliliter of broth and considered to be resistant if more than this amount was required.

RESULTS AND DISCUSSION

The results are presented in Table 1. Sixteen of the 26 coagulase-positive strains were non-typable or resistant to lysis by all the phages (diluted or undiluted). Two strains exhibited the pattern 80/81. This is of considerable interest, since published

TABLE 1

COAGULASE-POSITIVE STAPHYLOCOCCI ISOLATED FROM HUMAN MOUTH

Patient No.	Strain No.	Phage Patterns Obtained upon Typing Strain with Routine Test Dilution of 10^{-3}–10^{-5} Dilution of Each Phage	Phage Patterns Obtained upon Retyping Same Strain with Concentrated Phage or 10^{-1} Dilution of Each Phage	Minimal Inhibitory Concentration of Antibiotic (μg. or Units/Ml)		
				Penicillin	Oleandomycin	Oxytetracycline
12	1	NT*	NT	0.39	0.78	0.19
14	2	NT	NT	0.39	0.78	0.19
15	3	NT	NT	0.39	1.56	0.39
16	4	NT	NT	0.39	0.78	0.19
18	5	NT	NT	0.39	0.78	0.39
22	6	NT	NT	0.39	0.39	50
28	7	NT	NT	0.39	1.56	50
34	8	NT	NT	0.39	0.39	25
35	9	NT	NT	0.78	1.56	25
40	10	NT	NT	1.56	0.78	0.39
49	11	NT	NT	6.25	0.39	12.5
52	12	NT	NT	0.78	1.56	0.19
55	13	NT	NT	0.39	0.78	0.19
56	14	NT	NT	0.39	0.78	0.19
57	15	NT	NT	3.12	0.78	25
60	16	NT	NT	1.56	0.78	12.5
64	17	NT	42D	0.78	1.56	0.19
70	18	NT	29/79	0.78	1.56	0.19
71	19	NT	42D	0.78	0.78	0.19
78	20	80/81 (E.S.)†	.....	12.5	1.56	100
82	21	80/81 (E.S.)	.....	100	0.78	100
84	22	3A/3B/3C/55/71	.....	12.5	0.78	12.5
93	23	52/52A	.....	0.78	1.56	0.19
140	24	42D	.....	0.39	0.78	0.19
167	25	29/42D/52A/53/54/75/77/83	.....	0.78	0.78	25
200	26	6/47/53/54/75/81/83	.....	1.56	0.78	12.5

* NT—non-typable or resistant to lysis by all the phages comprising the typing set.

† (E.S.)—also designated as "type 80/81"; 52/42B/81; 52/42B/80/81; 52/42B/81/44A; frequently referred to as "epidemic strain"; however, other strains may be equally as pathogenic.

reports[10] have referred to *S. aureus* of phage type 80/81 as a major cause of infections in many parts of the world. Type 42D has been frequently cultured from mastitis in cows and from dairy products,[11] whereas we[12] have isolated the other types from the noses and throats of asymptomatic human carriers upon their admission to a hospital. It should, however, be emphasized that all coagulase-positive staphylococci are potential pathogens and, regardless of their phage types, may under certain conditions be virulent and cause disease.

Of the 25 coagulase-positive strains, 18 were susceptible to oxytetracycline, 25 to penicillin, and all to oleandomycin. Susceptibility and/or resistance to the antibiotics tested appeared to be independent of the bacteriophage type of the organism. These strains were more susceptible in vitro to both penicillin and oleandomycin when compared with previous data[13] obtained upon assaying staphylococci isolated from clinical material in a general hospital.

In comparison, the present findings are somewhat similar to those reported by Taplin and Goldsworthy.[7] Of the 79 coagulase-positive strains studied by these investigators, less than half (30) were non-typable with bacteriophage, and approximately one-fifth (15) were resistant to penicillin.

We have, therefore, through this limited study, obtained additional data concerning the bacteriophage patterns and antibiotic-susceptibility of a number of coagulase-positive strains of *S. aureus* which have been isolated from the mouth.

SUMMARY

In the present investigation, 26 coagulase-positive strains of *S. aureus,* isolated from the oral cavity of a total of two hundred individual patients, were phage-typed and also tested for susceptibility to a number of currently available antibiotics. Most of these strains were non-typable or resistant to lysis by all the phages. Of this number, 18 were susceptible to oxytetracycline, 25 to penicillin, and all to oleandomycin.

We wish to thank Dr. Frederick C. Fink, Chas. Pfizer and Company, for co-operation rendered during the course of this laboratory study.

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Blood-Volume Measurements of Dog Teeth

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There is a renewed interest in the vascular system of the tooth. Some characteristics of the vascular channels of the dental pulp have recently been investigated by Cheng and Provenza.[1] In addition, direct microscopic observations of the living pulp tissue have been reported by others.[2–4] Because of the extremely small blood volume and inaccessibility of the sample, no data have been reported on the vascular capacity of the tooth.

A previous report from this laboratory described a method for the determination of the blood volume of extracted dog teeth by utilizing radioisotopes.[5] The primary purpose of the present investigation was to compare the blood volumes obtained for extracted teeth with those of *in situ* teeth. A number of procedures for the determination of the *in situ* blood volume of the tooth was examined. The most practical method appeared to be quick freezing of the teeth and jaw by direct application of liquid nitrogen, thereby fixing the vascular content of the dental pulp.

In order to evaluate the blood-volume measurements of freshly extracted teeth, comparisons were made between (1) blood volumes obtained from freshly extracted contralateral teeth in the same dogs; (2) blood volumes of contralateral teeth frozen *in situ;* and (3) blood volumes of freshly extracted teeth and contralateral teeth, frozen *in situ.*

MATERIALS AND METHODS

Mongrel dogs of both sexes were used in these studies. The dogs were anesthetized with a combination of morphine sulfate and sodium pentobarbital. Radioiodinated (I^{131}) serum albumin (human) RISA,* which was passed through an ion-exchange resin to remove free iodide, was injected intravenously via the femoral vein in each of the dogs. A precalculated amount of radioactivity was administered, so that each milliliter of the dog blood would contain at least 300,000 detectable counts (approximately 5 microcuries RISA per kilogram). Thirty minutes were allowed for adequate mixing of the RISA in the circulation. A known volume of venous blood was obtained, and the extraction of the teeth was initiated. Forceps were used for most of the extractions. In some instances, because of the difficulty encountered in delivery of the tooth, a flap and alveolectomy were performed to avoid fracture of the tooth. In spite of all precautions, fracture of the roots occurred occasionally, and those teeth were discarded. The extracted teeth were root-planed to remove remnants of the periodontal

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* Abbott Laboratories.

ligament, wiped clean with gauze, and placed in suitable containers for determination of radioactivity.

The frozen *in situ* teeth were obtained by direct application of liquid nitrogen to the jaws of the anesthetized dog. The frozen jaws were resected by a vibrating saw.* The frozen resected jaw was kept on dry ice while the teeth were chiseled from the alveolar process. The teeth were prepared in the same manner as the extracted teeth and placed in a vial for counting while still frozen.

The blood volume of individual teeth was determined by comparing the radioactivity of a 1-ml. sample of blood with the radioactivity of a tooth according to the following equation:

$$\text{Blood volume of tooth (in ml.)} = \frac{\text{Total counts/tooth}}{\text{Counts/ml of whole blood}}.$$

Since I^{131} emits gamma radiation, it was not necessary to prepare the sample in any special way for the counting technique employed. A well-type scintillation gamma

TABLE 1

COMPARISON OF BLOOD VOLUMES OF TEETH FROM DOG MANDIBLES

(Volumes in Milliliters)

Tooth	Dog A		Dog B		Dog C	
	Right Extracted	Left Extracted	Right Frozen	Left Frozen	Right Extracted	Left Frozen
1. Central incisor	0.0004	0.0006	0.0019	0.0022	0.0017	0.0022
2. Intermediate incisor	0.0007	0.0008	0.0029	0.0035	0.0032	0.0034
3. Corner incisor	0.0049	0.0043	0.0050	0.0055	0.0053	0.0048
4. Canine	0.0414	0.0448	0.0257	0.0243	0.0238	0.0242
5. First premolar	0.0033	0.0035	0.0015	0.0016	0.0029	0.0033
6. Second premolar	0.0048	0.0051	0.0062	0.0062	0.0058	0.0068
7. Third premolar	0.0056	0.0052	0.0054	0.0052	.....	.....
8. Fourth premolar	0.0072	0.0075	0.0071	0.0066	0.0051	0.0043
9. First molar	0.0323	0.0277	.....	.....	.....	.....
10. Second molar	0.0032	0.0029	0.0068	0.0057	0.0044	0.0034
11. Third molar	.....	.....	.....	.....	.....	.....
Total volumes	0.1038	0.1024	0.0625	0.0608	0.0522	0.0517

counter was used to measure the radioactivity. Blood samples and the teeth were placed in individual vials which fitted into the well of the detector. All samples were counted for at least 5,000 counts and corrected for background.

RESULTS

The blood volumes of mandibular teeth of three selected dogs from which the greatest number of teeth was removed intact are presented in Table 1. For identification purposes, the teeth are listed consecutively from the mid-line for both left and right sides, thus facilitating the comparison of the values for contralateral teeth.

Values for dog A are the blood volumes of right and left paired, freshly extracted

* Stryker.

teeth. When the contralateral teeth in the mandible were compared, the percentage difference was greatest in the tooth with the smallest blood volume (tooth 1). Conversely, with the exception of tooth 9, the differences were less in teeth with larger vascular capacity. The per cent difference of total blood volume on the left quadrant when compared with freshly extracted teeth on the right quadrant was 1.35 per cent, or 0.0014 ml.

Values for dog B illustrate the blood-volume capacity of paired teeth which had been frozen *in situ*. Again, the difference in blood volumes of individual teeth was greater in those teeth with small vascular content. When the combined blood volumes of the teeth of the right and left quadrants were compared, the percentage difference was 2.7 per cent, or a blood-volume difference of 0.0017 ml.

The blood volumes of freshly extracted teeth compared with contralateral teeth frozen *in situ* are shown by the data of dog C. Contralateral paired teeth from dog C exhibited slightly more variation in blood volume than did those of the other two dogs. The total blood volume of teeth obtained by extraction compared with the total blood volume from the teeth of the frozen quadrant indicated a difference of 0.0005 ml., or 0.9 per cent.

DISCUSSION

The similarity in blood volumes of contralateral teeth as shown by these studies was not unexpected, since it would be reasonable to assume that the bilateral morphological symmetry of the dentition would include the vascular capacity of the tooth. The use of mongrel dogs of various ages negates the possibility of comparing the vascular capacity of teeth of different dogs in absolute values. It is possible, however, to compare the blood volume of paired teeth in the same dog.

The reliability of blood-volume values for extracted teeth depends on whether significant amounts of blood escape from the teeth during extraction. The fact that the total blood volume of the extracted teeth from the right quadrant of dog C was slightly greater than the frozen quadrant on the opposite side would indicate that the difference between these two sides is due to biological variation rather than to blood loss. Furthermore, the differences of total blood volume of the teeth from the frozen mandible of dog B were slightly greater than the difference obtained in the same comparison of freshly extracted teeth of dog A. One may conclude that these differences also confirmed the biological variations and presumably reflect the error of the measuring method.

The possibility that the radioiodinated serum albumin might enter the dentinal tubules also would tend to give values other than the true blood volumes; however, this may be assumed to be uniform for paired teeth. After extirpation of pulps from cuspid teeth, a residual amount of radioactivity was present in the hard structure. This finding indicated that there may be a movement of protein between plasma and dentinal fluid. Such transfer of protein into dentinal fluid has recently been demonstrated in the mouse.[6]

In the absence of more direct methods, it would appear that satisfactory measurements of the blood volume of teeth of experimental animals may be accomplished by utilizing freshly extracted teeth. The blood volume of a tooth in one quadrant could

serve as a control value for its pair mate in the contralateral quadrant. The determination of the blood volume of the tooth may provide a basis for the evaluation of factors affecting the vascularity of the pulp.

SUMMARY

Blood volume can be determined in teeth extracted from dogs after the systemic administration of radioiodinated serum albumin.

The blood volumes of paired extracted and *in situ* teeth are sufficiently similar to justify determinations on extracted teeth. The blood loss from the extracted tooth is less than can be detected by the methods employed in this study.

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The Cholesterol Content of Some Bovine and Human Oral Tissues

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This study was begun with the hope that a usable correlation might be found between the developmental status of the tooth and the cholesterol content of its pulp. Fisher, Belding, Opinsky, and Spinella[1] reported that endogeneous respiration was highest in this tissue when dentinogenesis was most active and that it dropped to much lower levels as dentin formation was completed. There was dissatisfaction, however, with the relatively crude anatomic criteria previously used for evaluating progress in tooth formation. It was believed that some pulpal constituent, subject to convenient analysis, might show progressive quantitative changes of sufficient magnitude during tooth maturation to provide a practical index of development. Previous studies in this direction by Riedesel and Fisher[2] and by Fisher, Wait, Chalup, and Nash[3] on the nitrogen content of the pulp failed to provide a solution to the problem.

Cholesterol was selected as a possibly useful constituent because cell nuclei were reported by Stoneburg[4] to be especially rich in this material and because the pulp is believed by some to become less cellular with age as its fibrous component becomes more abundant. Marinetti, Scarmuzzino, and Stotz[5] found that 1.02 per cent of the lipids that constituted one-third of the dry weight of cytochrome oxidase was cholesterol.

Progress in the analyses of bovine dental pulp eventually showed that the original objective of the study would not be achieved. It was therefore extended to include other tissues for the purpose of expanding basic comparative data, since the cholesterol content of oral structures has received only limited attention. Hodge[6] found the mean cholesterol content of human gingiva to be 0.20 per cent of its moist weight. He also found that its mean value in adult tissue was slightly higher than for infants. Hess, Lee, and Peckham[7] reported the cholesterol content of human dentin and enamel to be 0.024 and 0.008 per cent, respectively. Hawk, Oser, and Summerson[8] state that the cholesterol content of normal saliva ranges from 2.5 and 9.0 mg. per 100 ml., and that "increases in cholesterol and in lipide phosphorus have been noted in certain dental disorders."

This investigation was pursued with full knowledge of the ambiguity of the term "cholesterol" and the difficulties in analyzing tissues specifically for cholest-5-en-3β-ol. It was intended to obtain comparative quantitative information about that group of substances ordinarily referred to as "cholesterol" and that are characterized by precipitation with digitonin and by participation in the Lieberman-Burchard reaction.[9]

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MATERIALS AND METHODS

The tissues included in the present study were bovine dental pulp, gingiva, parotid gland, Achilles tendon, and human dental pulp. The bovine tissues were obtained from freshly slaughtered animals that were approximately two years old. The teeth and their pulps were classed as *embryonic* if root formation was not yet evident, as *young* if root formation was in progress, and as *mature* if root formation was complete, following the practice of our previous studies.[1, 3] The pulps of human teeth were classified on a similar basis. The teeth from which they were obtained were non-carious and were extracted for orthodontic or other therapeutic considerations. The fresh tissues were frozen for preservation immediately after isolation and were later thawed out when and as required for analysis. Tissue samples were adjusted to 100 mg. of moist weight. Individual bovine pulps often provided more than one sample, but in the case of human pulp it was necessary to pool the tissue from several teeth in order to obtain a single sample.

TABLE 1

CHOLESTEROL CONTENT OF SOME BOVINE ORAL TISSUES

(Per Cent of Moist Weight)

Tissue	No. of Samples	Mean Per Cent	S.D.	Range
Dental pulp				
Embryonic.....	30	0.067	0.006	0.049–0.088
Young.....	30	0.069	0.007	0.052–0.097
Mature.....	30	0.078	0.007	0.062–0.102
Gingiva.....	30	0.112	0.035	0.071–0.147
Parotid Gland.....	30	0.242	0.007	0.214–0.282
Achilles tendon....	22	0.039	0.003	0.019–0.052

The analytical method relied on the well-known Lieberman-Burchard reaction. The specific procedures of Foldes and Wilson[10] were employed, these being modifications of the micromethod described by Schoenheimer and Sperry.[11] Analyses were accomplished with a spectrophotometer* equipped with double thermospacers to maintain the environmental temperature of the reaction cuvettes at 25° C. Absorption measurements were made at 620$\mu\mu$.

RESULTS

The analytical results for cholesterol in bovine tissues are presented in Table 1. Those for human dental pulp are shown in Table 2. Data on bovine Achilles tendon are included with those for bovine oral tissues, to provide some comparison with dense collagenous connective tissue, since connective tissue is important in the constitution of dentin, pulp, and gingiva.

DISCUSSION

While the mean cholesterol content of dental pulp increases slightly with increasing maturity, it is evident that the change is not great enough to provide a practical measure of the developmental status of the tooth. The slightly higher concentration of

* Beckman Model D.U.

this material in the pulps of more mature human and bovine teeth is similar to Hodge's observations respecting human gingival tissue. Salit and O'Brien[12] believed that "the contention of several investigators that the cholesterol content increases with age" was corroborated by their own studies of the crystalline lens. Increase in sterol content with age has been reported also for the aorta and the costal cartilages.[9] The level of plasma cholesterol is known to rise with age in human subjects.[9] In a study of lipids of serum and connective tissue of rats and rabbits, Noble and Boucek[13] noted that the cholesterol concentration increased with the age of the tissues but that there was no significant correlation between it and the lipid values of the serum.

The higher cholesterol values for human dental pulps as compared with bovine pulp may possibly be explained by differences in actual age of the two sets of material and by species differences. The bovine teeth were from animals approximately two years old. The human teeth, on the other hand, were from persons in the second and third decades of life. Thus, if the possibility of species differences is excluded, the human pulp tissue was at least five times as old as the bovine tissue, and usually even older.

TABLE 2

CHOLESTEROL CONTENT OF HUMAN DENTAL PULP
(Per Cent of Moist Weight)

Pool No.	No. of	Class	Per Cent Cholesterol
1.....	7	Young and mature	0.163
2.....	10	Most mature	0.218
3.....	15	Mature	0.245

The possibility of species differences, however, cannot be dismissed entirely, although evidence in its favor is not overwhelming. Slight differences in total sterol of the whole body have been reported for rat, rabbit, dog, and cat and have been noted in the connective tissue of rat and rabbit.[9] In comparing human and bovine tissues only, liver, gall-bladder bile, and adrenal gland have been reported to show the higher concentrations of cholesterol in the human material.[9] A comparison of bovine and human[7] gingival tissue also shows the latter to be higher in cholesterol. The mean cholesterol content of human dental pulp appears to be approximately three times greater than in the bovine tissue.

The cholesterol content of human dentin reported by Hess *et al.*[8] is approximately one-tenth of that found in the human pulp reported in the present study. If the results are comparable, it suggests that only a small portion of the pulpal cholesterol is intimately associated with the constituents that ultimately form the organic matrix of the dentin.

SUMMARY

This report presents the results of spectrophotometric analyses of cholesterol in bovine dental pulp, gingiva, parotid gland, and human dental pulp. The mean values for bovine and human pulp were approximately 0.07 and 0.21 per cent of moist weight, respectively. Its presence in bovine gingiva amounted to 0.11 per cent of moist weight. The cholesterol content of bovine parotid gland was 0.24 per cent of its moist weight.

These findings, together with those of an older report on human gingiva, indicate that the cholesterol content of pulp and gingiva increases slightly with age and also that the values for human pulp and gingiva are higher than those for the corresponding bovine tissues.

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*In Vitro Calculus Formation**

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It has been generally assumed that dental calculus is formed by spontaneous precipitation of salivary calcium phosphate.[1–6] The concept presented by Neuman[7] that calcification in bone and teeth is a result of seeding or epitaxy may, however, also apply to dental calculus.

This possibility was considered by Wasserman, Mandel, and Levy,[8, 9] who studied in vitro calcification of plaque material. They assessed calcification of the plaque by microradiographic procedures and by examination of histologic sections stained with alizarin red-S and the Von Kossa silver method. It was found that the plaque calcified after 4 days' exposure to a calcifying solution. Of a number of enzymes tested for inhibition of calculus formation, only hyaluronidase was effective when added to the calcifying solution. From this finding they concluded that a carbohydrate-protein complex in the plaque was involved in initiating the calcifying process.

However, their method was not sufficiently sensitive to detect minute quantities of calcium phosphate in the plaque; therefore, it is possible that calcification could have been prompted by crystalline calcium phosphate deposited in the plaque before exposure to the calcifying solutions, since it is known that hydroxylapatite crystals can act as templates and bring about mineralization. It is not unlikely that the plaque employed by Wasserman *et al.*[9] contained calcium phosphate because it was obtained from the mouths of calculus formers and because calcification occurred in this material even when hyaluronidase was present in the calcifying solution.

The present paper describes an improved in vitro method[10] for developing calculus by which it is possible to study independently plaque formation and calcification and to assess calcification by quantitative microchemical procedures. This method was used to study the importance of carbonate and pH on the rate of calculus formation and to determine the calcifying ability of plaque free of inorganic calcium phosphate.

EXPERIMENTAL METHODS

Plaque was produced on small paper disks which were rotated and thus given alternate exposure to saliva and air. The disks, 5 mm. in diameter, were punched from filter paper‡ which had previously been treated with a spray coating.§ Individual disks were mounted between two stainless-steel pins held together by spot welding. The

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free ends of the pins were inserted in a piece of tygon tubing which had been mounted on a glass rod (Fig. 1). The rod was positioned over a plastic trough containing saliva, through which the disks rotated at 1 rpm. At this speed, the disks were exposed to saliva for about 20 seconds and to air for 40 seconds per cycle.

Paraffin-stimulated whole saliva was collected each morning under mineral oil in polyethylene funnels. These were provided with clamps on the stem and were kept in a water bath during collection, either at 37° C. or in ice water. The saliva was pooled under oil in a beaker and stirred by a magnetic device before being transferred to the trough.

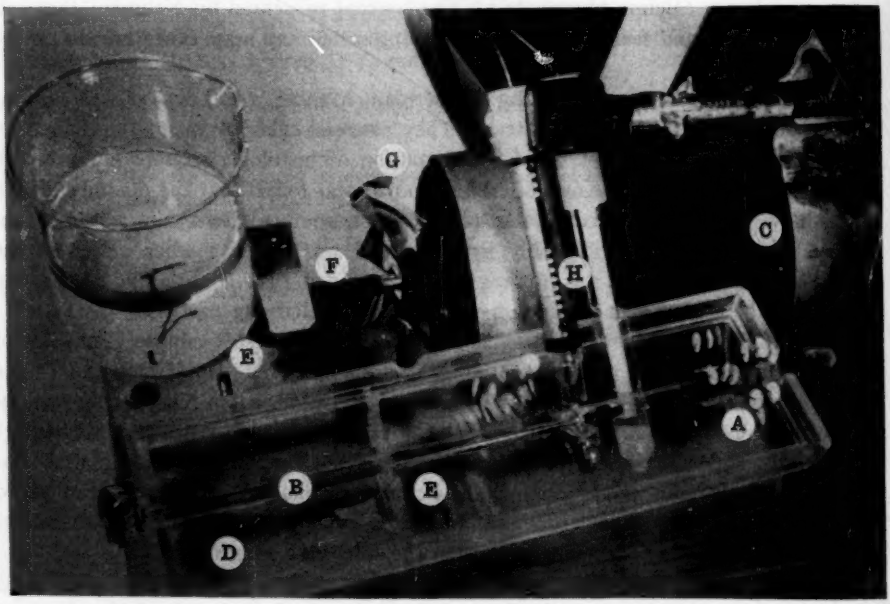

FIG. 1.—Apparatus for in vitro plaque formation. Paper disks mounted on steel pins (*A*). Glass rod (*B*) connected to low-speed gear shaft on motor (*C*). Plastic trough (*D*). Rubber tubing connecting salivary beaker and trough (*E-E*). Screw clamp connected to motor (*F*). Fan (*G*) and electrodes (*H*).

Plaque formation was carried out for 3 hours, as previously described, in an incubator at 37° C. The disks were then washed in distilled water for 2 minutes and transferred in groups of six to eight into stoppered vials containing 50 ml. of calcifying solution. After exposure to this medium for 21 hours at 37° C., the disks were removed and washed as before. The whole procedure was repeated daily for intervals, up to 3 weeks for some groups of disks. Control disks were either exposed to saliva for 3 hours and kept moist in the incubator during the remaining 21 hours or exposed to calcifying solution only.

The calcifying solution[11] was made up free of CO_2 and was composed of $CaCl_2$, 16.0×10^{-4} M/L — (6.40 mg/100 ml Ca); KH_2PO_4, 28.8×10^{-4} M/L — (8.93 mg/100 ml P); barbital, 0.02 M/L; KCl, 0.143 M/L; and KOH, 0.012 M/L. The pH = 7.40, and $\mu = 0.165$.

Since it was found that during incubation saliva lost CO_2 and increased in pH as much as 1 pH unit during incubation, experiments were designed to counteract these effects. In one study the CO_2 pressure was maintained at a level of 60 mm. Hg during incubation, the same as that reported for saliva.[6] This was accomplished in a modified vacuum oven,* with built-in thermostatically controlled heating element and closely fitted door. The beaker containing pooled saliva under oil was connected with rubber tubing to the trough (Fig. 1). An ordinary screw clamp, attached with rubber tubing to the motor axle, prevented saliva from entering the trough until the motor was started. Thus saliva was not exposed to the atmosphere in the oven until the proper gas mixture had been established. Under the guidance of a mercury manometer, part of the air was evacuated from the oven by a vacuum pump, and the desired amount of CO_2 gas was injected. A fan on the motor assured proper mixing. Control samples were taken with a 50-ml. syringe and analyzed for CO_2 in a manometric apparatus.† Concentrations were kept at 8 ± 0.5 per cent throughout the experiment. CO_2 analyses were also performed on salivary samples at the beginning and end of each exposure; pH readings were taken during the experiment with a shielded glass electrode and a calomel electrode, which were mounted in the trough and connected to a potentiometer.‡

In another experiment performed in air, salivary pH of 6.8 was maintained during plaque formation by bubbling a stream of air first through concentrated HCl and then through the saliva.

In order to measure calculus formation, the disks were washed and dried at room temperature, removed from the assembly, and dried to constant weight in vacuum at 60° C. The weight of the disks was determined on a semimicro balance§ having a sensitivity of 0.02 mg. The weight of the deposit was obtained by subtracting the weight of the untreated disks. Calcium and phosphorus analyses were performed on each disk, except in cases with extremely small amounts of deposits, when several disks were pooled. The minerals were dissolved in 0.5 ml. of 3 N HCl and diluted to 10 ml., from which aliquots were taken for duplicate analysis. Calcium was determined by volumetric titration with EDTA, using eriochrome black as indicator.[12] Phosphorus was determined colorimetrically as molybdo-vanado–phosphoric acid,[13] by means of a spectrophotometer.||

In addition to the chemical assessment, histologic examinations were undertaken. Undecalcified formalin-fixed histologic sections were prepared from some of the disks with deposits. In addition to hematoxylin-eosin staining, tolouidine blue and Von Kossa silver staining were used. Some of the sections were subjected to microradiographic and polarizing microscopic examination.

The calcifying ability of the organic matrix was tested in a series of experiments after the minerals had been eliminated as a possible initiating factor. Disks with artificial calculus were demineralized in saturated EDTA at pH 7.0 for 24 hours. Since

* Forma-Scientific Inc., Marietta, Ohio.

† Van Slyke, A. H. Thomas Company, Philadelphia, Pa.

‡ Beckman, type G, Beckman Instruments Inc., Fullerton, Calif.

§ Mettler, E. Mettler, Zurich, Switzerland.

|| Beckman DU, Beckman Instruments Inc., Fullerton, Calif.

the decalcified plaques tended to wash off the disks during handling, one group was fixed in 10 per cent neutral formalin for 10 minutes before it was demineralized. This treatment has been shown not to affect calcifiability.[9] No Ca or P could be detected in the EDTA-exposed plaques.

After thorough washing, the decalcified disks were placed in calcifying solution for 1 week, washed, and subjected to chemical analysis.

RESULTS

The weight as well as the mineral content of each disk increased with the number of exposures, showing a straight-line relationship with time. Ca and P values for two comparable experiments carried over a 4-day period are plotted in Figure 2. Each

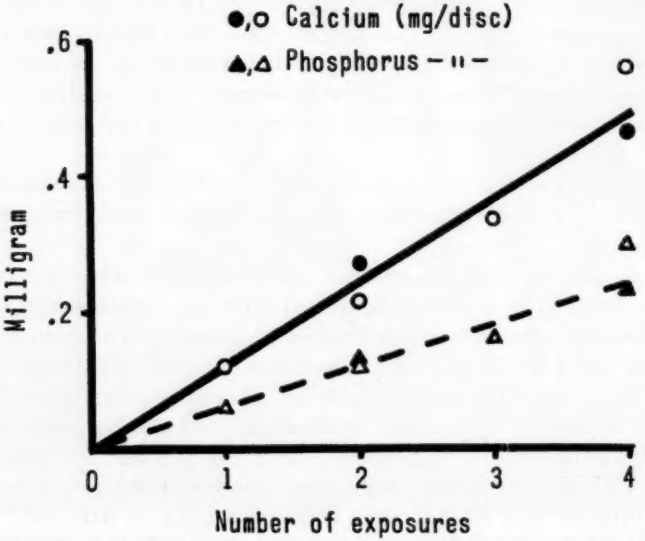

FIG. 2.—Deposition of minerals during formation of artificial calculus. Comparison of two experiments employing regular exposures in air.

point on the graph represents the mean of six to eight measurements. The average increase in Ca per day was 125 μg., and the Ca/P ratio was consistently 2.0. Similar findings were observed in experiments continued for 3 weeks.

In the control groups the disks exposed to only calcifying solution showed no calcification, while disks exposed to saliva alone brought about only minute mineral deposition.

It is significant that, once some mineral had deposited, calcification would continue in calcifying solution alone. Figure 3 shows an experiment in which further weight increase of a certain amount of calculus was accomplished by exposure to calcifying solution only. However, the average daily Ca uptake was only 79 μg.

Microscopic examination of undecalcified sections showed distinct layers in the deposit, which were related to the number of salivary exposures. With tolouidine blue staining, metachromasia was observed in the matrix. Von Kossa silver staining, as

well as microradiographs, showed deposition of mineral only in the matrix and not within the paper of the disks. The polarizing microscope revealed orderly orientation of crystals in well-mineralized specimens.

In the previous experiments plaque formation was carried out in air, and the salivary pH increased from 7.2 to 8.2 during the 3 hours' incubation. When the pH level was maintained between 7.0 and 7.2 by incubating under increased CO_2 pressure (60 mm. Hg), there was a marked decrease in calculus formation. Controlling the pH by bubbling with gaseous hydrochloric acid also decreased the amount of deposit, but the inhibitory effect was less than that of CO_2. These findings are given in Figure 4. The control figures were obtained on plaque material which had been formed without pH

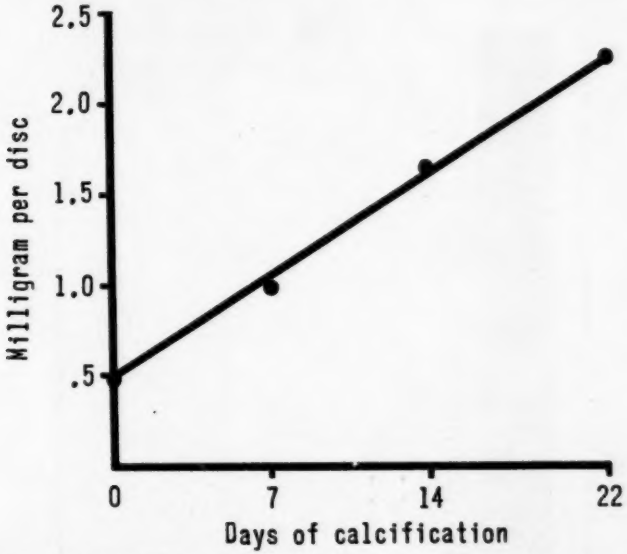

FIG. 3.—Exposure of calcified plaque to calcifying solution only. The mineral uptake is here demonstrated as increase in the total weight.

regulation. In this control series, more mineral was deposited than in the first experiments because saliva was collected and inserted in the incubator at 37° C. instead of at 0°, in order to facilitate accurate pH readings.

Table 1 shows that considerable amounts of Ca and P were deposited in the completely demineralized matrix. The Ca/P ratio, 1.9, was approximately the same as that of deposits obtained in previous experiments. The greater amount of mineral obtained on the formalin-fixed than on the non-fixed disks is probably due to the presence of more organic matrix on the former.

DISCUSSION

Although the saliva employed in the present study was collected from non-calculus formers, calcium phosphate was consistently deposited in the plaque under the conditions of this experiment. Plaque produced in vitro retained its calcifying ability even after rigorous decalcification. In agreement with previous reports,[9] it therefore appears

that a calcifying factor must be present in the organic matrix, even when formed from saliva of non-calculus formers.

From other work now under way there is some indication that this factor may be associated with micro-organisms. This is also supported by the finding of calcified bacteria in natural calculus, as observed by electronmicroscopy of undecalcified material.[14] Intracellular formation of hydroxylapatite in certain unicellular organisms has previously been reported[15] and must therefore be considered as a possibility in calculus formation. But further work is needed to clarify this problem.

There are several mechanisms by which calcification of calculus could theoretically take place.

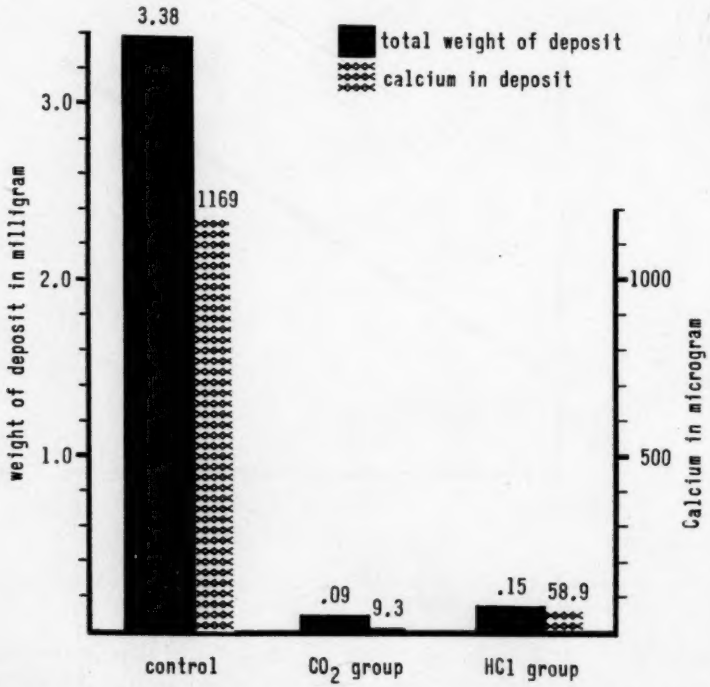

FIG. 4.—Effect of CO_2 and pH on in vitro calculus formation. Comparison after eight exposures of total weight and calcium content of deposits.

TABLE 1

MINERALIZATION OF DECALCIFIED PLAQUE

(Average Weight and Mineral Content per Disk)

	Weight (Mg.)	Calcium (μg.)	Phosphorus (μg.)
Before decalcification	3.80	1,315	.....
After decalcification	0.08	0	0
After recalcification	0.53	113	60.5
Formalin-treated and recalcified	1.33	381	204

1. Formation of $CaHPO_4 \cdot 2H_2O$ follows increased activity product of Ca^{++} and $HPO_4^{=}$ ($a_{Ca^{++}} \cdot a_{HPO_4^{=}}$) to the point of spontaneous precipitation.[11] The secondary calcium phosphate would then undergo transformation to form hydroxylapatite which could be deposited in the plaque. Despite several attempts, it has not been adequately demonstrated that this reaction takes place in saliva. If such a mechanism was contributing during regular plaque formation, it is not likely to have taken place when the pH was controlled.

2. Even below the point of spontaneous precipitation, formation of hydroxylapatite may take place if a suitable template is available. Hydroxylapatite itself, as well as certain collagens, has such a trigger effect.[11, 16] Available evidence seems to indicate that saliva is normally supersaturated with respect to hydroxylapatite and therefore could favor such a mechanism. Our results could indicate that a template of organic nature was present in the organic matrix.

3. It is interesting to speculate on a third possibility, which may be nothing but a variation or combination of the two former mechanisms. Organic components in the plaque and micro-organisms may under certain conditions have the ability to absorb and increase the local concentrations of calcium and phosphate to the point of starting crystallization. Bone matrix has been shown to remove preferentially calcium from solutions,[16] and dental plaque is claimed to have higher calcium content than would be expected from salivary concentrations.[17]

4. Decomposition of bacterial, cellular, or salivary elements within the plaque could bring the pH up locally to the point where spontaneous precipitation would take place. Such a process would not necessarily be associated with viability of micro-organisms.[9]

Based mainly on our observation of remineralization, it appears that the increased CO_2 tension, as well as the lowered pH, affected the "organic calcifying factor." Further work is needed to characterize this component. The method developed seems suitable for the study of calculus formation and has the advantage that deposits are rapidly formed and can be quantitatively assessed. Matrix formation and calcification can be studied separately.

SUMMARY

An in vitro technique is reported which permits independent study of plaque formation and calcification on a quantitative basis.

Maintenance of salivary CO_2 at 60 mm. Hg and pH below 7.2 during plaque formation inhibited in vitro formation of calculus. Because decalcified calculus was shown to remineralize, it was concluded that the organic matrix contained a substance which could initiate calcification.

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Interrelations in Dental Development. I. Interrelationships within the Dentition

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At the present time, as a result of recently published data,[1, 2] it is possible to make a quantitative evaluation of the extent to which a posterior mandibular tooth is advanced or retarded in its formation or eruption. Such a determination, however, is of limited value until its predictive significance is known. One may ask whether advancement or retardation in the formation of one tooth is necessarily indicative of the developmental status of other teeth and whether dental advancement at one age is necessarily predictive of advanced tooth development at a later stage.

Similar questions pertain not only to individual teeth but to particular morphological classes of teeth and to particular stages of development. Is the tooth an independent unit of development? Are morphological classes (here molars or premolars) consistent within themselves as to developmental status but inconsistent between tooth classes? Are particular stages of development, such as beginning calcification or apical closure, consistent across the dentition but relatively independent of other stages of somatic development? The answers to these questions clearly bear on the complex problem of the local determinants of dental development.

Obviously, answers to these questions can be obtained only by serial, longitudinal analyses of developing dentitions, since in no other way can the processes of development be followed within individual children. Having such data on a sizable sample of clinically healthy, native-born American white boys and girls, we have examined in considerable detail the interrelationships, during growth and development, of the successive stages of calcification and eruptive movement of the mandibular molar and premolar teeth.

MATERIALS AND METHODS

The present investigation was based on the age at appearance of specified stages of tooth calcification and tooth movement as ascertained from over three thousand serial, longitudinal oblique-jaw radiographs, supplemented by lateral head plates of 259 white Ohio-born participants in the Fels Longitudinal Program.[1-3]

Data on three calcification stages (beginning calcification, crown completion, and apical closure) and two stages of tooth eruption (alveolar eruption and attainment of the occlusal level)[3, 4] were converted into normalized *T*-scores[5] and punched on standard 80-column IBM cards. Intercorrelations were then obtained for each pair of variables representing different stages of development of premolars and molars (P_1–M_3). Of the total number of correlations, the majority were calculated with the aid of an electronic computer,* and the remainder were computed with a desk calculator.

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* IBM model 602.

In analyzing the matrix of correlations, special attention was focused on the two morphological classes (M and P), separately on specific teeth (P_1, P_2, M_1, M_2, and M_3), and, finally, on particular stages (beginning calcification, etc.), using the mean value of r obtained from the mean z-transform of r.[6] In this way the partial autonomy of anatomical areas and developmental stages could be considered.

For simplicity, correlations involving a particular tooth or morphological class are described as *intratooth* (or class) correlations, as contrasted with correlations involving different teeth or classes or stages, which are called *intertooth* (or class or stage) correlations. Since the repeated use of data on the same individuals obviously limits the independence of the individual correlations, exact tests of significance of differences between groups of correlations have not been attempted.

RESULTS

At the beginning of the study it was not known whether correlations involving boys and girls could be combined or whether sex-specific correlations would have to be employed throughout. Accordingly, individual correlations were calculated for boys and girls separately and then compared, using a simple sign test.

For 78 comparable sex-specific correlations involving different stages of tooth development, girls were higher than boys in 45 and tied in 1. Against the chance hypothesis (39:39), the value of chi-squared was 1.28, not significantly different from zero at the 5 per cent level of confidence. Moreover, the mean value of 78 intercorrelations was only 0.05 higher in the girls. Accordingly, the use of combined-sex correlations employing sex-specific normalized T-scores was both justifiable and yielded the maximum possible sample sizes.

For a total of 210 combined-sex intercorrelations given in matrix form in Table 1, individual values of r ranged from 0.90 to 0.96 (for events closely linked in time), through zero or even negative values (usually with very small sample sizes). The mean value of r for all intercorrelations was 0.42, indicating a moderate degree of relationship among developmental events covering a 13-year period separated by an average of 6 years.

However, since the matrix comprising the raw correlations did not lend itself well to analysis by tooth, by developmental stage, by morphological class, or intervening time, such analyses were accomplished by averaging individual correlations involving the variables concerned. Thus, as shown in Table 2, 145 correlations involving tooth calcification alone (mean $r = 0.46$) and 52 involving eruptive tooth movement (mean $r = 0.56$) were higher than 104 correlations involving both tooth formation *and* tooth eruption (mean $r = 0.30$). This finding may be taken to indicate partial autonomy of the two developmental processes—tooth calcification and tooth movement.

In very much the same fashion, correlations involving a given tooth, that is intratooth correlations, were higher than correlations involving different teeth (P_1 versus M_1, P_1 versus M_2, etc.) (mean $r = 0.55$) than interclass correlations (M with P), where the mean r was 0.40.

Exactly the same pattern emerged when developmental stage was taken into account. For the five developmental stages involved, but ignoring morphological class, the total of intrastage correlations was again higher (0.57) than with interstage cor-

TABLE 1

TOOTH INTERCORRELATION MATRIX*

Tooth	Stage	P_1 CC	P_1 AC	P_1 AE	P_1 OL	P_2 BC	P_2 CC	P_2 AC	P_2 AE	P_2 OL	M_1 CC	M_1 AC	M_1 AE	M_1 OL	M_2 BC	M_2 CC	M_2 AC	M_2 AE	M_2 OL	M_3 BC	M_2 CC
P_1.....	Beginning calcification	0.42	0.39	0.17	0.15	0.34	0.27	0.30	0.33	0.29	0.32	−0.08	0.06	−0.05	0.35	0.37	0.01	0.40	0.12	0.27	0.20
P_1.....	Crown completion (CC)		0.59	0.52	0.45	0.32	0.74	0.59	0.53	0.51	0.44	0.34	0.16	0.17	0.22	0.52	0.39	0.33	0.32	0.19	0.37
P_1.....	Apical closure (AC)			0.52	0.39	0.34	0.37	0.77	0.57	0.61	0.39	0.52	0.46	0.25	0.27	0.40	0.61	0.29	0.23	0.39	0.07
P_1.....	Alveolar eruption (AE)				0.96	0.05	0.38	0.12	0.77	0.63	0.28	0.46	0.03	−0.04	0.10	0.35	0.02	0.56	0.68	0.24	−0.20
P_1.....	Occlusal level (OL)					−0.05	0.29	0.12	0.72	0.62	0.19	0.37	0.27	−0.09	0.08	0.26	−0.06	0.50	0.43	0.12	−0.80
P_2.....	Beginning calcification (BC)						0.60	0.56	0.43	0.52	0.25	0.25	0.35	0.26	0.48	0.47	0.36	0.38	0.46	0.47	0.33
P_2.....	Crown completion							0.62	0.61	0.59	0.45	0.39	0.19	0.25	0.43	0.61	0.45	0.37	0.35	0.43	0.52
P_2.....	Apical closure								0.71	0.69	0.46	0.63	†	†	0.39	0.61	0.69	0.45	0.58	0.36	0.55
P_2.....	Alveolar eruption									0.95	0.32	0.53	0.04	†	0.22	0.42	0.53	0.71	0.66	0.23	−0.12
P_2.....	Occlusal level										0.18	0.32	−0.47	0.13	0.39	0.36	0.30	0.77	0.62	0.13	−0.39
M_1.....	Crown completion											0.56	0.47	0.48	0.38	0.70	0.63	0.41	0.21	0.21	0.51
M_1.....	Apical closure												0.55	0.42	0.29	0.63	0.84	0.47	0.43	0.41	0.36
M_1.....	Alveolar eruption													0.94	0.24	0.50	0.28	0.89	0.81	0.24	0.30
M_1.....	Occlusal level														0.14	0.45	†	0.64	†	0.34	0.22
M_2.....	Beginning calcification															0.70	0.59	0.56	0.53	0.68	0.36
M_2.....	Crown completion																0.73	0.68	0.53	0.54	0.75
M_2.....	Apical closure																	0.49	0.48	0.52	0.63
M_2.....	Alveolar eruption																		0.90	0.33	−0.12
M_2.....	Occlusal level																			0.29	0.52
M_3.....	Beginning calcification																				0.75

* Combined-sex correlations using sex-specific normalized T-scores.

† Small sample correlations not given.

relations (mean $r = 0.42$), suggesting some degree of local autonomy for the stage of development.

Not shown in Table 2, although the raw data are given in the first tabulation, intratooth correlations were carefully analyzed and compared. For the five mandibular teeth (P_1–M_3) individual mean intercorrelations were 0.40, 0.46, 0.42, 0.50, and 0.30, respectively. Although the second premolar and molar (P_2 and M_2) are known for their variability both in timing and in sequence, they were not notably different from the average; they could not be said to exhibit special independence during their development and movement toward the occlusal level.

Since this extensive analysis of interrelationships included some correlations involving events closely linked in time, as, for example, beginning calcification of P_2 and M_2, and other events separated by as much as 13 years (for example, beginning formation of M_1 and apical closure of P_2), there was the obvious question of how the

TABLE 2

MEAN INTERCORRELATIONS FOR CALCIFICATION AND MOVEMENT OF MANDIBULAR MOLAR AND PREMOLAR TEETH

Correlations	No. of Correlations*	r†
Tooth calcification vs. tooth calcification	145	0.46
Tooth movement vs. tooth movement	52	0.56
Tooth calcification vs. tooth movement	104	0.30
Intratooth correlations	11	0.60
Intertooth correlations	67	0.45
Intraclass correlations	36	0.55
Interclass correlations	42	0.40
Intrastage correlations	22	0.57
Interstage correlations	56	0.42

* Number of separate correlations, *not* number of subjects.
† Mean r from mean z-transforms of r.

average value of the correlations changed with increasing elapsed time. Therefore, the correlations were grouped according to the *average elapsed time between events* and examined for trend; that is, correlations involving events separated by 5 years were averaged, those involving events separated by 5.5 years were averaged, etc.

This procedure of averaging correlations according to the elapsed time between the events involved yielded the results shown in Figure 1. As might be expected, short-term correlations exhibited the highest mean values, with averages of from 0.5 to 0.7, and individual correlations as high as 0.96. With increasing elapsed time between the events involved, the average of the correlations dropped but was still systematically higher than zero after a period of 13 years (Fig. 2).

Since no comparable intercorrelation matrix involving developmental events could be found in the literature, an intercorrelation matrix was generated for the number of ossification centers of the hand and wrist at successive age intervals, using a series of 170 boys and girls selected to exclude like-sex siblings. The number of ossification centers was utilized as representing discrete, but irreversible, events, much as with successive stages of tooth formation. The similarity between the two correlation/elapsed-time graphs is obvious, even though the wrist-hand centers complete them-

selves earlier (Fig. 2). In essence, the predictability of tooth status, while decreasing with increased elapsed time between events, is reasonably comparable with that of other developmental processes characterized by discrete happenings and is considerably lower than predictability for developmental processes involving size and size increments, such as stature or weight.

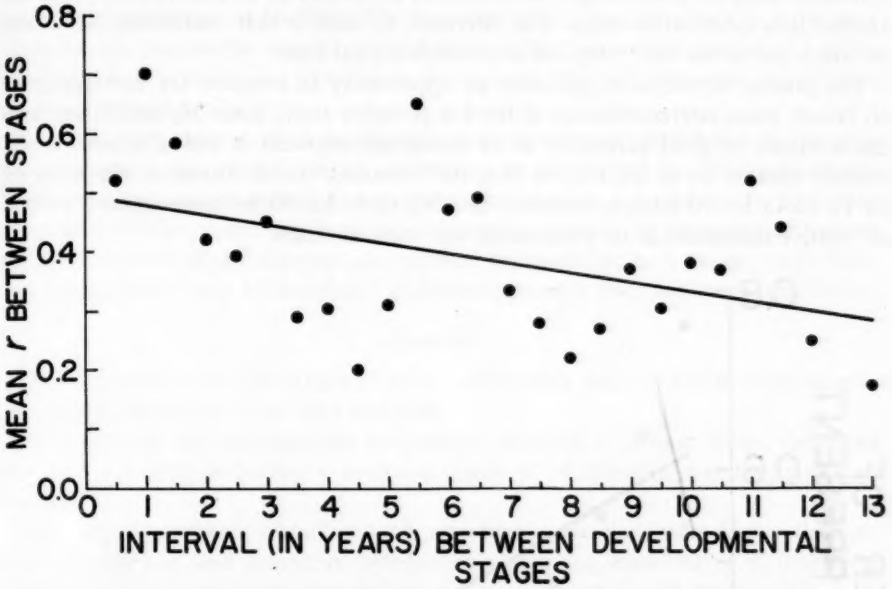

FIG. 1.—Decrease in predictive efficiency with increasing intervals between successive stages of tooth calcificaton and movement. With increasing time between the events represented by the correlations (here given as averaged values), over-all predictability decreases but remains significantly better than zero even after a 13-year period.

DISCUSSION

As is evident in the preceding section, developmental events within the dentition prove to be moderately intercorrelated during the growing period in both sexes. This association holds true for successive developmental stages involving initial calcification, crown development, and apical closure. It is true for tooth movement, and it is true for the relationship between tooth formation and tooth movement. Moreover, although the magnitude of the intercorrelations decreases with advancing time span between the developmental events involved, there is predictability over a full 13-year period.

As might have been expected, correlations within a tooth (intratooth correlations) are higher than correlations between teeth (intertooth correlations), thus reaffirming the tooth as the prime developmental unit. In similar fashion, morphological class has meaning developmentwise, at least within the two morphological classes considered here, for intraclass correlations average higher than interclass correlations. To some extent, therefore, a "field" concept is applicable, despite the very marked differences in the maturity rates of either premolar or molar teeth.

That intrastage correlations are higher than interstage correlations follows logically; but this fact is, as yet, devoid of biological meaning. Is it possible that there are factors regulating or timing the attainment of particular *stages* of formation or movement, even though such stages may occur 9 years apart? Confirmatory evidence exists from the fact that tooth calcification and tooth movement are both more highly correlated *intra se*, even though, timewise, one tooth may be in a movement stage and another in a calcification stage. The inference, at least, is that controlling factors exist which cut across both temporal and morphological lines.

The present investigation provided an opportunity to compare the communalities or, rather, mean intercorrelations of the five posterior teeth. Since M_2 and P_2 are both characterized by great variability as to timing and sequence, it was of interest to determine whether P_2 or M_2 was, in fact, the "aberrant" tooth. However, the mean r's for P_2 (0.46 for 90 intercorrelations) and M_2 (0.50 for 90 intercorrelations) yielded no positive indication as to which tooth was more aberrant.

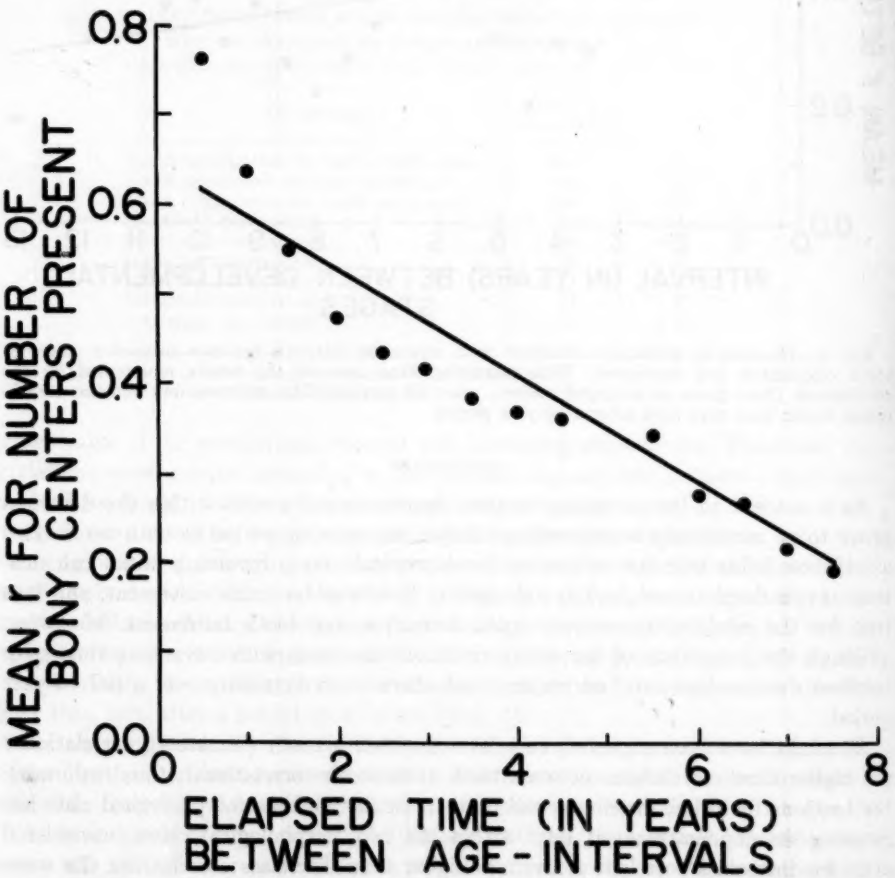

FIG. 2.—Decrease in predictive efficiency with increasing intervals for number of hand-wrist ossificaton centers. In both magnitude and age trend the predictive efficiency of the hand ossification and tooth calcification are comparable.

One of the purposes of the present study was to determine whether, from a single oblique-jaw plate, one could predict the future dental status of an individual or generalize about the probable course of dental development of a group. As to the former, caution is indicated, except for short-term predictions involving a few years at most. Stature at age eighteen is far better predicted by stature at age two than dental status at thirteen is predicted by dental status at seven. On the other hand, and especially for groups, some degree of predictability does exist. Using specific events as a measure of comparison, such as the number of hand-wrist ossification centers present at a given age, predictability within the dentition is of the same order of magnitude as predictability in the hand.

It follows, then, that *marked* developmental acceleration or retardation in the dentition at an early age is predictive of acceleration or retardation at a later age. As applied to the two morphological classes here considered and especially to particular teeth, predictability is necessarily higher. However, it would be erroneous to assume that uniform rates of development characterize the dentition as a whole; early moderate advancement may be associated in individuals with later moderate retardation.

SUMMARY

Interrelationships for five stages of tooth calcification and movement were investigated in 259 white Ohio-born boys and girls.

For a total of 190 combined-sex correlations covering a 13-year range, the mean value of r was 0.42, indicating a moderate degree of relationship over the time span involved.

Expectably, intratooth, intraclass, and intrastage correlations were higher than intertooth, interclass, and interstage correlations, suggesting some degree of functional autonomy of individual teeth, morphological classes, and stages of development.

The mean intercorrelations for the five teeth considered were of the same order of magnitude: neither P_2 nor M_2 was conspicuously lower in communality.

Predictability decreased from approximately 0.6 over a 1-year period to approximately 0.2 after 13 years had elapsed, thus resembling predictability values for the number of hand-wrist ossification centers.

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Assessment of Dental Caries Increments
I. Construction of the R.I.D. Index

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"The total increment of cavities, or incidence, in a test period is the safest criterion for judging caries activity. An index which fails to include all units affected can provide an incorrect estimate of the total increment."[1] Although many indexes have been presented, none of these will permit comparison of dental caries increments of children of all ages regardless of dentition.[3-5, 7, 9, 14, 15, 17] This comparable quality was a requirement in a longitudinal study of children undertaken in 1953 for the purpose of correlating dental caries activity with certain laboratory tests.[2] An index was needed which would express dental caries activity in such a manner that the rates of children in primary, mixed, and permanent dentitions could be compared.

The purpose of this paper is to present an index of the Relative Increment of Decay (R.I.D.) designed to allow comparison of dental caries increments of children with dissimilar dentitions and hence suitable for the longitudinal assessment of dental caries activity of an individual or group, regardless of age.

EXPERIMENTAL METHODS

Construction of the index.—In order to make comparisons of increments of dental caries activity between individuals or groups of individuals, certain information must be known: the period of observation or time, the number of units or surfaces showing change, and the number of units or surfaces which were available or at risk during the interval. The general principle underlying the construction of any index of the time rate of change of decay (or of any other variable) must be based solely on observations made at some time *a* and at some later time *b* and upon the time interval from *a* to *b*.

The surface is taken as the basic unit of observation. A grosser unit would be the tooth itself, a finer unit the anatomic area[3] or lesion.[4, 5] What is argued here, based on surfaces, would apply equally to these other units.

At a given time, a surface is classified as being in one and only one of four possible states: (1) present and decay-free, (2) present and decayed, (3) present and filled, (4) missing.

The index rate of decay is based on observations of individual surfaces at time *a* and time *b*, and the increase in decay is based on a count of the surfaces which have changed from one state to another. The sixteen categories of possibilities for any sur-

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face experience in a given time interval, as measured by an examination at the beginning and end of the interval, are shown in Table 1.

The individual boxes in Table 1 contain the number (N) of units representing status at the two times. The N's are labeled, e.g., N_{1-3}, to facilitate the location by row and column. The entries in this table are, for any individual, counts of the number of surfaces falling into each of the sixteen possible categories of status at times *a* and *b*. For example, in the first row and first column would be the number of surfaces (N_{1-1}) which were present and decay-free at time *a* and which were also present without decay at time *b;* in the fourth row and third column would be the number of surfaces not present at time *a* which had erupted and been filled by time *b* (N_{4-3}).

The count of surfaces falling in the box N_{2-1} is a count of surfaces which represent reversals. The method of handling these is not pertinent to the argument presented here for the system of measuring the increment of dental caries activity. The management of this category will be discussed in a later paper.

TABLE 1

SIXTEEN CATEGORIES OF POSSIBILITIES FOR SURFACE EXPERIENCE IN A TIME INTERVAL

Status at Time *a*	Status at Time *b*			
	Decay-free	Decayed	Filled	Missing
Decay-free...	N_{1-1}	N_{1-2}	N_{1-3}	N_{1-4}
Decayed.....	N_{2-1}	N_{2-2}	N_{2-3}	N_{2-4}
Filled.....	N_{3-1}	N_{3-2}	N_{3-3}	N_{3-4}
Missing.....	N_{4-1}	N_{4-2}	N_{4-3}	N_{4-4}

Any index must be built from these counts: first, a measure of the *absolute increment of decay* (surfaces becoming carious), then a measure of the *number of surfaces available for decay* during the interval. Their ratio is the relative increment of decay during the interval *a–b*. Dividing by the length of the interval *a–b* will yield the *relative increment of decay per unit time,* which is used as the index of rate of decay.

Absolute increment of decay.—Only five of the counts of surfaces in this table give information as to the increment of decay: N_{1-2}, N_{4-2}, N_{1-4}, N_{4-3}, and N_{1-3}. These are the counts of the surfaces which went from decay-free or missing to decayed during the interval, the counts of the surfaces which went from decay-free to filled or missing, and the counts of those which went from missing to decayed. Of these, only the first two—N_{1-2} and N_{4-2}—give positive information of new decay. The other counts might indicate new decay, but surfaces could fall into these categories for reasons other than decay; for example, a surface could be filled because of decay in adjacent surfaces, or a surface could go from decay-free to missing because of exfoliation or extraction with no decay.

The indication of possible decay given by the number of surfaces going from decay-free to filled (N_{1-3}) should not be ignored, but, if included in a measure of the increase in decay, these counts should be weighted less than the two (N_{1-2} and N_{4-3}) giving certain information of new decay. Boyd, Cannon, and Lighton[6] and Hill and Kniesner[7]

recommended a weight factor for surfaces changing from caries-free to filled. Boyd *et al.* reported a 25 per cent increase in caries experience due to fillings alone.[6]

The occlusal surface is the surface most likely to be filled in a tooth when that surface is not carious, because of the inclusion in the restoration of proximal carious lesions. Random sampling of 157 examinations of children, aged six to seventeen years, from the Washington State School for the Deaf revealed 2,307 carious lesions involving occlusal and/or proximal surfaces. Of these 2,307 carious lesions, 587 represented teeth which had involvements of the proximal surfaces to the exclusion of the occlusal surfaces. Assuming that all 2,307 carious lesions had been restored, there would have been 2,894 surfaces restored. Hence 587 of the surfaces thus counted would have been involved in restorations but would not have been carious. A weighting factor was calculated by the ratio of

$$\frac{\text{2,307 actual carious surfaces}}{\text{2,894 assumed restored surfaces}} = 0.797$$

(99 per cent confidence limits on this weight factor was 0.77–0.81).

For convenience in the computations, the weighting factor for surfaces changing from caries-free to restored was rounded off to 0.8. Having determined this weight in an objective manner, it was held constant in this study for all computations for surfaces showing change from caries-free to restored. It should be recognized that the weighting factor is subject to error, in that it was estimated on the basis of occlusal surfaces alone. However, it was reasoned that this is the surface most likely to be restored for reasons of prevention or extension and also is the surface most readily estimated.

The count of the number of surfaces going from decay-free to missing also gives uncertain information as to new decay. If the time interval between successive observations is short, the appropriate weight given to this count would be so small that the count can be ignored completely. Bruckner, Hill, and Wollpert[5] suggested a similar management of such surfaces in incremental study. Hence surfaces going from decay-free to missing are not included in the data because of the lack of information concerning the condition of the surfaces at the time of extraction or exfoliation.

Surfaces appearing in the interval of time between examinations and becoming filled ($N_{4\text{-}3}$) are given full weight because of the unlikelihood of such surfaces being filled without evidence of caries in the short time interval. This situation as described is assumed to represent a high degree of caries activity. A tooth was considered erupted and available for decay when the occlusal surface was completely exposed.

Because of the foregoing rationale, the following is suggested as the measure of absolute increment to decay in the symbols of Table 1:

$$\text{Absolute increment of decay} = N_{1\text{-}2} + N_{4\text{-}2} + (0.8)\, N_{1\text{-}3} + N_{4\text{-}3}\,.$$

Surfaces available for decay.—To be counted in this category, a surface must either have been present without decay at the beginning of the interval between *a* and *b* or have erupted during the interval. There is no way of determining from the observations at the beginning and end of the interval when a surface erupted during the interval. In the absence of such information, it seems best to count such surfaces as present for half the interval or, alternatively, to count them as half-surfaces present for the whole interval. In this measure of the number of surfaces available for decay, it is desirable

to use only those surfaces which could possibly have contributed to new decay during the period.

The number of surfaces available for decay is measured by totaling the number present without decay at time *a* plus half the number of surfaces which erupted during the interval *a–b*. In the symbols of Table 1:

$$\text{Surfaces available for decay} = N_{1\text{-}1} + N_{1\text{-}2} + N_{1\text{-}3} + \left(\frac{N_{4\text{-}1} + N_{4\text{-}2} + N_{4\text{-}3}}{2}\right).$$

The ratio of these two measures (*absolute increment of decay/surfaces available for decay*) multiplied by 100 is the Relative Increment of Decay (R.I.D.) in the interval *a–b*. The constant of 100 is used to provide a whole number for convenience in reading:

$$\text{Relative increment of decay} = \frac{N_{1\text{-}2} + N_{4\text{-}2} + (0.8)\, N_{1\text{-}3} + N_{4\text{-}3}}{N_{1\text{-}1} + N_{1\text{-}2} + N_{1\text{-}3} + (N_{4\text{-}1} + N_{4\text{-}2} + N_{4\text{-}3})\,/\,2} \times 100.$$

Bodecker,[3] Henschel and Lieber,[4] James and Parfitt,[8] and Lefkowitz and Venti[9] have all emphasized the importance of time in expressing increments of dental caries activity. It should be noted that the interval of time utilized here is 6 months and that the increment is expressed as the activity for that period. Any interval can be used, so long as it is presented.

DISCUSSION

Many indexes have been designed to measure the prevalence and incidence of dental caries, in order to permit the appraisal of dental needs.[1, 10] Methods have also been devised for clinical testing, in order to measure increments of dental caries.[1] Some investigators have utilized the number of carious lesions per tooth in an interval as the index for comparison,[4, 5] and others the change in the size of the lesion.[11, 12] The most widely used measure is the DMF index,[13] adapted to increment study.

Where the DMFS or DFS index is utilized in assessment of dental caries activity in children, it is important that the ages and dentitions of the children be similar, to limit error because of different tooth populations. The DMFS index is, by definition, suited for the permanent dentition and represents serious limitations in dealing with changing dentitions.[4] Although the def index[15] is adapted to the deciduous teeth, the use of the two measures does not permit comparisons of children with dissimilar dentitions. In an effort to circumvent the problem of a double measure in children in mixed dentition, some investigators have considered the changes occurring in the permanent teeth alone and have excluded the deciduous teeth.[1] However, information regarding the child's caries activity is then excluded, and question may be raised in regard to the comparative quality of these children with children in complete permanent dentition.

The R.I.D. index was designed to permit the comparison of dental caries increments of children of any age or type of dentition. By using this tool, the rates of dental caries activity of children in mixed dentition can be compared with those of children in primary or permanent dentitions. The R.I.D. index can be used in longitudinal studies because it is an expression of the total dental caries activity in given time intervals.

Like most instruments utilized for measuring purposes, the R.I.D. has disadvantages:

a) The expression is a pure number and not a descriptive number, as is the DMF index.

b) The expression is a figure with a decimal which may not seem meaningful.

c) The R.I.D. index presupposes, as do most other indexes, that each surface has an equal likelihood of becoming carious. Many surfaces are unlikely ever to become carious in some individuals and are prone to caries in others.[1] Barr, Diodote, and Stephens[16] showed wide variability in the distribution of dental caries on different surfaces and areas and suggested analysis of caries attack by the location of the new lesions.

d) The R.I.D. index does not take into consideration filled surfaces which became carious at subsequent examinations in this study. Because of the complications arising in this assessment when the surface is the unit of measure, these changes were not included. If the index utilized the anatomical area described by Bodecker,[3] inclusion of this assessment would be more feasible for certain surfaces on certain teeth. The index does provide a weighting factor for surfaces changing to filled without certainty of decay.

The need of an expression of dental caries activity in an absolute figure which allows comparison of dental carious increments of the four-year-old with the seven-, eleven-, or fifteen-year-old is met by the R.I.D. index. This unique feature alone justifies its use for studies when such values are desired.

SUMMARY

An index is presented for assessing the relative increment of decay in intervals of time. The index is an expression of the ratio of the new carious surfaces to the total surfaces available in a given time interval. Weighting factors are utilized for surfaces changing from caries-free to filled and from missing to carious or filled. Some of the limitations of the index are mentioned.

The R.I.D. index is designed to permit comparisons of rates of dental caries activity of children in primary, mixed, and permanent dentitions or to allow longitudinal assessment of the dental caries rates of the child throughout the period of active growth.

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Assessment of Dental Caries Increments
II. A Comparison of the R.I.D. and DMFS Indexes

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Clinical tests for the efficacy of dental caries control measures require an equitable means of comparing dental caries increments. Sound comparisons must consider the total activity in terms of time and the units at risk, as well as the development of new lesions. If all these factors are not considered, then comparisons are not reasonable.

Children of grade-school age are most suitable for clinical caries tests because they are available as a group for testing periods and they have newly erupted tooth surfaces. However, a problem in the reporting of the findings of children in mixed dentition arises because of the deciduous teeth. Consequently, the deciduous teeth are frequently omitted from consideration of dental caries increments because of convenience in data manipulation. When this practice is used, the estimates of the total increments cannot be expected to be comparable with the findings of different studies utilizing different age groups.[1]

The purpose of this paper is to demonstrate how the Index of the Relative Increment of Decay, the R.I.D. index, compares with the DMFS index used in increment studies for children in mixed and permanent dentitions. Furthermore, an attempt will be made to show how the R.I.D. allows comparisons of dental caries increments of children of different ages and different dentitions, i.e., deciduous, mixed, and permanent.

MATERIALS AND METHODS

The data utilized for this study were obtained from dental examinations conducted at 6-month intervals over a 3-year period at the Washington State School for the Deaf.[2] The examinations were performed by one individual, using dental mirrors, explorers, a dental light, a dental chair, compressed air, and posterior bite-wing radiographs. A single recorder indicated the findings in accordance with the method described by Klein and Palmer.[3] The posterior bite-wing radiographs taken by a dental hygienist were read subsequently and the information added to the charts.

The data for the children under study were grouped according to the type of dentition—mixed or permanent. Random selections for one 6-month interval were made for the purpose of comparing measures of dental caries increments for the two groups (Table 1).

Additional data for three separate age groups were selected from the total study

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material, in order to permit comparison of measures of longitudinal dental caries increments. Selection was based on age at the beginning of the study, character of the dentition, and completeness of records (Table 2).

RESULTS

A comparison of the increment of dental caries activity of forty children in permanent dentition, expressed in terms of the R.I.D. and the increase or change in DMFS, termed "Δ DMFS," is shown in Figure 1. A coefficient of correlation of 0.93 demonstrated the relationship between the R.I.D. and the Δ DMFS for a 6-month interval. A higher coefficient of correlation of 0.97 was shown to exist between the R.I.D. and

TABLE 1

SAMPLES SELECTED FOR COMPARISON OF MEASURES OF DENTAL CARIES INCREMENTS

Type of Dentition	No. in Sample	Age Range (Years)
Mixed	67	7–15
Permanent	40	10–18

TABLE 2

SAMPLES SELECTED FOR COMPARISONS OF MEASURES OF DENTAL CARIES INCREMENTS FOR 3-YEAR PERIOD

Group	No. in Sample	Age (Years)	Type of Dentition at First Examination
I	8	7– 7.5	Predominantly deciduous
II	17	10–10.5	Mixed
III	12	13–13.5	Predominantly permanent

the ratio of Δ DMFS to the number of available surfaces in Figure 2. These scattergrams demonstrate the high correlation coefficient computed between these two indexes for children in permanent dentition.

The comparison of the caries increments, measured by the R.I.D. and Δ DMF indexes, of the sixty-seven children in mixed dentition is shown in Figure 3. A coefficient of correlation of 0.70 was calculated. The degree of relationship measured by this lower correlation coefficient is illustrated by the amount of scatter in Figure 3.

A comparison of the two indexes of dental caries activity through a prolonged period where the nature of the dentition was changing from primary to permanent is demonstrated in Figure 4. Here is shown in a longitudinal manner what is suggested by the scattergrams of Figures 1–3. The two indexes were very closely correlated quantitatively where the dentitions were permanent. In the earlier years, in mixed dentition, the Δ DMFS presented a lower measure of dental caries activity than what was actually taking place as indicated in the R.I.D. index. The spread of the two curves decreased with time or maturity of the dentitions.

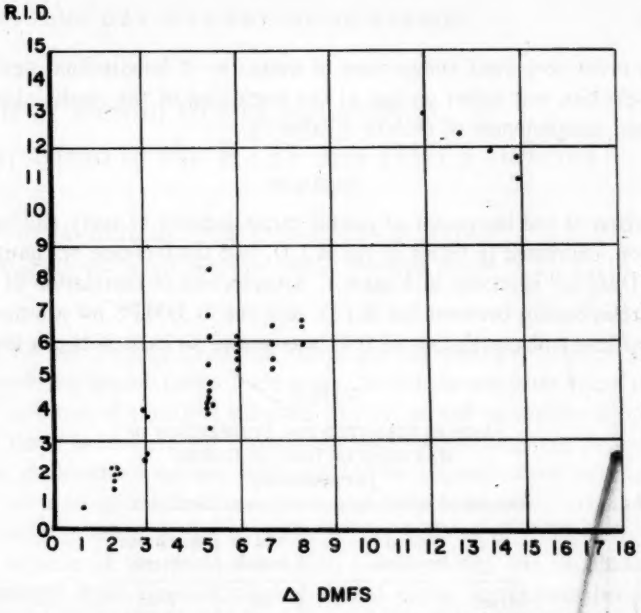

FIG. 1.—Scattergram showing the relationship of the R.I.D. and Δ DMFS indexes as measures of dental caries in permanent dentition. Δ DMFS = increase in DMFS. Coefficient of correlation = 0.93. *Conditions:* 6-month increment, 40 children, mean age 13.4 years.

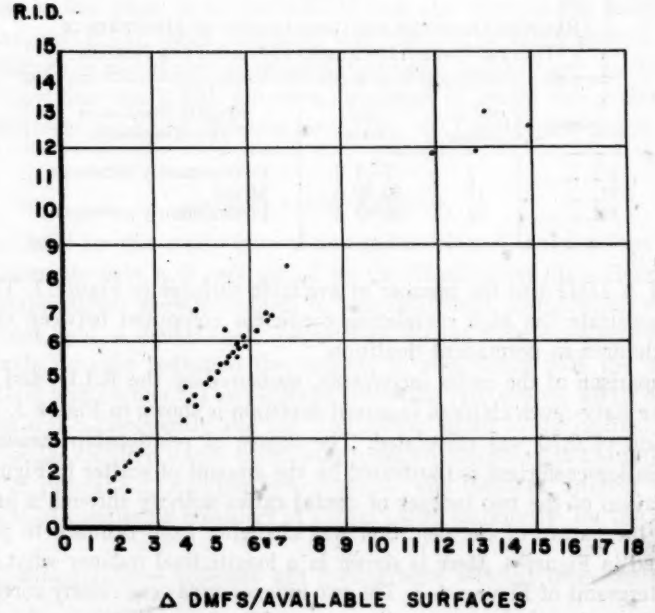

FIG. 2.—Scattergram showing the relationship of the R.I.D. and the ratio of Δ DMFS to surfaces available as measures of dental caries in permanent dentition. Coefficient of correlation = 0.97. *Conditions:* 6-month increment, 40 children, mean age 13.4 years.

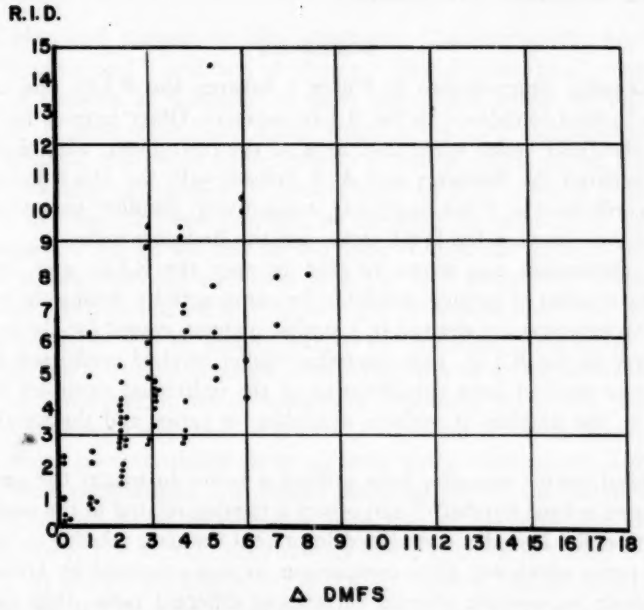

FIG. 3.—Scattergram showing the relationship of the R.I.D. and the Δ DMFS indexes as measures of dental caries in mixed dentition. Coefficient of correlation = 0.70. *Conditions:* 6-month increments, 67 children, mean age 10.3 years.

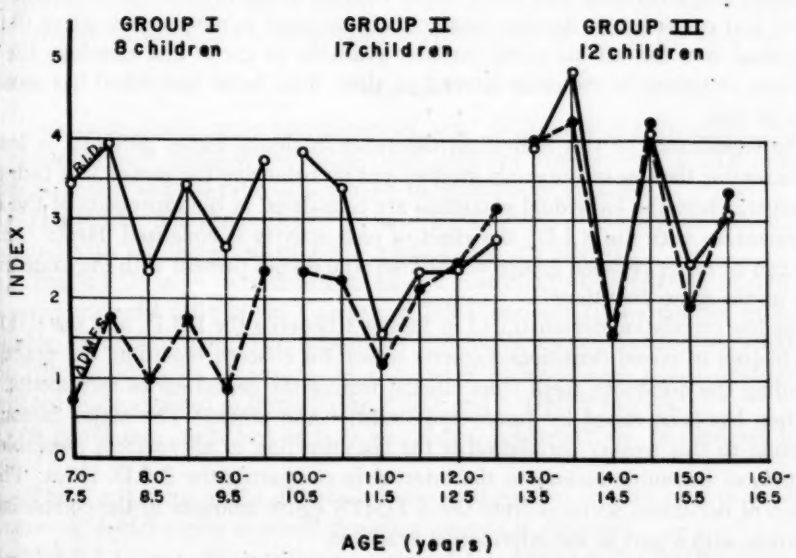

FIG. 4.—Relationship of R.I.D. and Δ DMFS indexes in changing dentitions. Each point represents the arithmetic mean for the children in each group for the examination intervals indicated.

DISCUSSION

The relationship demonstrated in Figure 1 between the R.I.D. and the Δ DMFS would seem to lend confidence to the R.I.D. measure. Other indexes have been compared with the DMF index when used as a survey instrument, with similar findings. Jackson[4] compared the Bodecker and ACF indexes with the DMF index and found correlation coefficients of 0.943 and 0.937, respectively. Sandler[5] showed a correlation coefficient of 0.87 between the DMF index and the Bodecker index.

A closer relationship was shown to exist between the R.I.D. and the ratio of Δ DMFS to the number of surfaces available for caries activity during the interval (Fig. 2). These two measures are derived in a similar manner, except for the weighting factors considered in the R.I.D. This somewhat higher level of confidence demonstrates the value to be derived from consideration of the individual variables of each child measured, i.e., the number of surfaces available for caries and the weighting factors utilized.

Other clinical testing measures have utilized a factor to weight the caries measure. Some have used a fixed number[6, 2] and others a number related to the number of teeth present.[8–10] The R.I.D. index is designed to present the data relative to the individual reactions in terms which will allow comparison, as was suggested by Dunning.[11]

As individuals accumulate affected surfaces at different rates, their potentials for caries diverge. In Figure 1, one of the extremes was a sixteen-year-old who had five surfaces become carious in a six-month interval and had relatively few surfaces available. The R.I.D. for individuals with Δ DMFS readings of 5 are noted to be quite different. The individual who has a major number of his tooth surfaces carious or restored and continues to develop more carious surfaces is more caries-active than the individual who has all his tooth surfaces available to caries and develops the same numbers of lesions in the same interval of time. This latter individual has more surfaces at risk.

The significance of this individual difference in dental caries potential is lessened by increasing the size of the group studied and by balancing the groups in a test study. However, where the individual potentials are considered in the expression of the caries increment, as with the R.I.D., the effect of past activity is equalized. Hence comparisons can be made between groups of children who do not present with the same experience or the same dentitions.

The low correlation demonstrated in Figure 3 between the R.I.D. and the Δ DMFS for children in mixed dentitions suggests reason for concern regarding the practice of excluding the deciduous teeth from clinical testing for dental caries increments. This question has been raised by James and Parfitt[12] and others.[1] The major factor contributing to this weaker correlation is the consideration of all surfaces available and all surfaces becoming carious in the interval in computing the R.I.D. index. The exclusion of deciduous surfaces from the Δ DMFS figure amounts to the comparison of the whole with a part of the information available.

The report, *Clinical Testing of Dental Caries Preventives,* of the American Dental Association states that "the total increment of cavities in a test period is the safest criterion for judging caries activity. An index which fails to include all units affected can provide an incorrect estimate of the total increment."[1] The results of the com-

parisons shown lend support to this statement. Considering the high correlations shown in Figures 1 and 2 and that of Figure 3, the advisability of comparing DMFS increments of children as dissimilar as these two groups would be questionable. Yet it is desirable to have some index which would allow these comparisons. The R.I.D. presents a common expression of increments of dental caries, giving consideration to all surfaces available and surfaces becoming involved in an interval of time.

Inasmuch as there has been considerable emphasis on longitudinal study, both for clinical testing and for growth and development studies, the material in Figure 4 was arranged to demonstrate the usefulness of R.I.D. as a longitudinal measure of dental caries activity. As the children in Groups I and II become older, the Δ DMFS index approaches the R.I.D. Put in another way, as the dentition of the group becomes more homogeneous, the Δ DMFS becomes more dependable as an expression of total dental caries activity. However, if one is desirous of comparing the dental caries activity of the children in Group I with that of Group II or Group III, some other measure must be utilized which gives consideration to all the activity taking place. The R.I.D. index does allow these comparisons. The dental caries activity of the individual children of the group in Figure 1 can be compared with that of the individual children or the group of Figure 3. The dental caries activity of the children in Group I at seven years of age (Fig. 4) are comparable with the activity rates of the children in Group II or Group III if the R.I.D. index is used.

The cyclic nature of the plots in Figure 4 are noted. Although these fluctuations are disconcerting, they are not deemed pertinent to the argument presented here. As explanation, they may be related to the examiner, to the 6-month interval, to the fact that the environmental circumstances varied for the odd intervals when the children were away from the institution for the summer months, or to the size of the samples.

SUMMARY

1. Six-month dental caries increments of forty children with permanent dentitions are presented and compared by using three different indexes—the R.I.D. index, the Δ DMFS index, and the ratio of Δ DMFS to the surfaces available.

a) The R.I.D. index was shown to have a coefficient of correlation of 0.93 when compared with the increment of DMF surfaces for the interval.

b) The R.I.D. index was shown to have a coefficient of correlation of 0.97 when compared with the ratio of the increase in DMF surfaces to the number of surfaces available for dental caries. The reasons for this higher correlation are discussed.

2. The dental caries increments of sixty-seven children with mixed dentitions are presented and compared in terms of the R.I.D. and Δ DMFS indexes.

a) A correlation coefficient of 0.70 was shown between these two indexes for this group.

b) The significance of the difference between these two indexes used to express increments of dental caries in mixed-dentition periods is discussed.

3. The R.I.D. was shown to have a close relationship with the DMF surface increments for individuals with permanent dentitions over a 3-year period. The relationship between these two indexes for a 3-year period for children in the mixed-dentition period was shown to be similar in pattern but different in the total measure of caries activity.

4. The R.I.D. index is suggested as an instrument to express dental caries increments in a manner which will allow comparison of increments of dental caries of individuals or groups of different ages and different dentitions, i.e., deciduous, mixed, and permanent.

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The Analysis of Calcium in Unashed Saliva

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The interference of magnesium and phosphate in calcium determinations has been eliminated by the use of calcein, EDTA, and a back-titration after an addition of excess EDTA.[1-6] Ericsson,[7] using Eriochrome Black T and a forward titration with EDTA, found it necessary either to ash saliva or to precipitate the proteins in saliva by acidulation. A rapid method of directly analyzing unashed serum and urine has been developed by using calcein and EDTA in a forward titration.[8]

This paper describes a rapid and accurate method of determining calcium in unashed saliva with calcein as an indicator, an excess of ethylenediaminetetra-acetate (EDTA) as the calcium-complexing agent, and a standard calcium solution for back-titration.

MATERIALS AND METHODS

0.04 M EDTA.—14.90 gm. of disodium dihydrogen ethylenediaminetetra-acetate dihydrate were dissolved in 4.5 ml. of 18 N NaOH and made up to 1 liter. A working solution of EDTA was 0.004 M.

Calcein solution.—2 gm. of calcein* were dissolved in 25 ml. of 1 N NaOH and made up to 100 ml. A working solution was prepared daily by diluting one twenty-fifth the stock calcein solution.

0.1 M calcium standard.—5 gm. of $CaCO_3$ were suspended in 50 ml. of water. 8 ml. of concentrated HCl were added and more, dropwise, until the solution cleared and then made up to 500 ml.

Digestion acid.—Equal parts of double vacuum-distilled 70 per cent $HClO_4$ and c.p. HNO_3 were mixed together.

Water used in the preparation of solutions and in rinsing glassware was demineralized by ion-exchange columns.†

All alkali used was carbonate-free.

Ashing.—0.5-ml. samples in 13 × 100-mm. Pyrex test tubes were oven-dried at 110° C. After adding 0.2 ml. of digestion acid, they were placed 1 cm. deep in metal blocks on a hot plate at 180° C. When a drop of clear acid remained, the tubes were placed on a 180° C. hot plate in a cylinder in which the tubes were immersed within a few millimeters of the top of the tubes. The tubes remained in the cylinder until the sample was a white ash.

Calcium analysis.—Two drops of a working calcein solution were added to each sample. 0.004 M EDTA was added in excess with a Krogh-Keyes syringe-pipette. Then

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* Frederick Smith Chemical Co., Columbus, Ohio.

† Ilico-way research model deionizers, Illinois Water Treatment Co.

1 N KOH was added and produced an orange-brown color. The sample was then back-titrated to a yellow-green end point with a standard calcium solution.

The titrations were performed in 13 × 100-mm. test tubes with a milk glass base as background, illumination by an overhead daylight bulb, and air stirring. The calcium solution was added from a mercury-controlled buret* with a capacity of 0.1 ml.

Decalcification of saliva.—1 gm. of a mixture of sodium oxalate plus a few milligrams of oxalic acid was added to 100 ml. of saliva, freshly collected in toluene. The oxalated saliva was allowed to stand at room temperature for 1 week. It was then centrifuged and filtered.

In Experiment 1, 5.0 ml. of saliva were collected from ten subjects by paraffin stimulation and centrifuged for 10 minutes. Duplicate 0.5-ml. samples were withdrawn from each directly for titration. Duplicate 0.5-ml. samples were also withdrawn for ashing. Both ashed and unashed samples were analyzed for calcium as described.

TABLE 1

ANALYSIS OF ASHED AND UNASHED SALIVA

Subjects	Ashed Saliva Ca Recovered (Mg.)	Unashed Saliva Ca Recovered (Mg.)	Difference (Per Cent)
A	0.0170	0.0168	1.1
B	0.0276	0.0278	0.7
C	0.0258	0.0258	0
D	0.0146	0.0150	2.6
E	0.0342	0.0344	0.5
F	0.0280	0.0278	0.7
G	0.0224	0.0226	0.9
H	0.0242	0.0242	0
I	0.0242	0.0246	1.6
J	0.0194	0.0190	2.1
Average			1.02

In Experiment 2, 100 ml. of saliva were decalcified and, upon analysis, were found to contain 0.0008 mg/ml; 0.5-ml. samples were withdrawn and ashed. On two consecutive days the following experiment was performed. Solutions containing, the range of milligrams of calcium normally found in saliva (4–8 mg. per cent) were added in duplicate to the unashed decalcified saliva and to the ashed decalcified saliva. The calcium solutions were added with a syringe-pipette calibrated at 0.5161 ±0.0001 ml. The ashed and unashed samples to which calcium was added were analyzed as described.

RESULTS

As shown in Table 1, the average per cent difference between calcium recovered from ashed and unashed saliva of the ten samples was 1.02, and neither was consistently greater.

As shown in Table 2, with decalcified saliva the average per cent recovered for 2 days was within 0.65 per cent of the amount added for ashed saliva and within 0.58 per cent for unashed. The recovery of calcium in the presence of oxalate from unashed

* Gilmont model, Manostat Corp.

saliva was undoubtedly due to the high affinity of calcium for calcein and the pH at which the titration was run.

All analyses were run in duplicate, and an average figure was used. The average deviation between duplicates was 1.1 per cent.

SUMMARY

The use of calcein as an indicator, EDTA as a calcium-complexing agent, and a back-titration with calcium is a rapid method of determining calcium in unashed saliva that is accurate to better than 1 per cent.

TABLE 2

RECOVERY OF CALCIUM ADDED TO DECALCIFIED SALIVA

	Ca Added (Mg.)	Ashed Saliva Ca Recovered (Mg.)	Unashed Saliva Ca Recovered (Mg.)
First day	0.0413	0.0414	0.0412
	0.0343	0.0346	0.0342
	0.0293	0.0292	0.0294
	0.0258	0.0256	0.0258
	0.0227	0.0230	0.0228
	0.0206	0.0204	0.0204
Av. deviation (per cent)		0.75	0.38
Second day	0.0413	0.0416	0.0414
	0.0343	0.0340	0.0344
	0.0293	0.0294	0.0290
	0.0258	0.0258	0.0256
	0.0227	0.0230	0.0230
	0.0206	0.0206	0.0204
Av. deviation (per cent)		0.55	0.77

The author wishes to acknowledge gratefully the helpful suggestions of Thomas B. Coolidge, M.D., Ph.D., during this study.

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Toxicity Studies of Cured Epoxy Resins

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Tissue-culture techniques have been used in almost every field of medicine and biology. It has been possible, for example, to study the chemical and physical effects on living cells and the specific action of drugs on the cells, along with the effects that antibiotics and antiseptics have on them. The tissue-culture technique is a sensitive, definitive, and quantitative technique that eliminates some of the shortcomings of animal testing, such as (1) differences between animals of the same species; (2) species differences, making accurate comparisons difficult; (3) quantity of animals required for adequate data; (4) partial masking of toxicity results by foreign-body reactions; and (5) inaccuracies of quantitative studies. In adapting the tissue-culture technique to dental materials, epithelial cells, which are the cells mainly encountered in the oral cavity, can be used.

A reasonable application of this technique was used in testing the toxicity of some of the newer epoxy resin formulations. Preliminary tissue-culture tests were conducted at the Wistar Institute, Philadelphia, Pennsylvania. Samples of cured epoxy resins from major resin producers were evaluated for toxicity along with samples of a commercial dental epoxy resin,* alumina-filled epoxy resins, and polymethyl methacrylate.

The epoxy resin samples were cured in teflon molds, ejected, and autoclaved for sterility. Triplicate samples were made for each manufacturer's resin. The resin strips were introduced into large tissue-culture flasks. The tissue-culture preparations were of two types. In the first type the tissue-culture medium was seeded with HeLa cells, and then the resin strips were introduced into the flask. In the second, the strips were introduced into a tissue-culture medium which had not been seeded with HeLa cells until after the strips were introduced. Controls identical with the experimental cultures were maintained for both types.

The culture medium was Eagle's (a buffered saline solution containing the essential ions in proper proportions) fortified with 10 per cent human serum. All cultures, both experimental and control, were sterile.

Those cultures exposed to the unfilled resin strips, the commercial epoxy resin, and polymethyl methacrylate showed no divergence from the controls within experimental error in seeding or cell count. It is apparent from these preliminary results that the resin strips tested were innocuous.

The alumina-filled epoxy resin strips inhibited the growth of cells and degenerated those cells used to seed the cultures. When this type of strip was introduced into established, healthy cultures, the sheeted cells and free cells degenerated within 48 hours. Furthermore, those cultures into which alumina-containing strips were introduced showed a progressive increase in pH from 7.0 to 11 during the period of 4 days, even though the culture medium was well buffered. The toxic effect of alumina occurred before the onset of any marked increase in pH, indicating that the pH was merely contributory to the toxicity.

Patch tests conducted on the flexor surface of the forearm of twenty dental students, using powedered, cured, unfilled resins and alumina-filled resins, showed neither signs of sensitization nor evidence of possible primary irritation.

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* Epoxylon, Surgident Ltd., Los Angeles, Calif.

The Endotoxic Activity of a Rat Oral Streptococcus

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Until recently it was assumed that endotoxic activity was restricted to the Gram-negative bacteria. Now it is known that reactions induced by endotoxins, such as the delayed skin response, local and generalized Shwartzman phenomena, febrile response, and the potentiation of epinephrine, are also elicited by lysates of Group A beta hemolytic streptococci (C. A. Stetson, *J. Exptl. Med.*, **104**:921, 1956). In view of the fact that streptococcal species have been shown to produce dental caries in gnotobiotic rats (F. J. Orland, *J. D. Res.*, **33**:145, 1954; R. J. Fitzgerald, personal communication), it seemed desirable to establish the possible endotoxic activity of such an organism.

Rat oral streptococcus strain FA-1 which induces dental caries in gnotobiotic rats was secured from Robert J. Fitzgerald, of the National Institute of Dental Research. The organism was maintained on microassay culture agar* containing added calcium carbonate and was cultivated on modified microinoculum broth for 48 hours at 37° C.

Streptoccocal lysate was prepared and employed according to the method of Stetson (*loc. cit.*), using pyrogen-free distilled water and physiologic saline. *E. coli* (026:B6) lipopolysaccharide* and physiological saline (0.15 M NaCl) were employed for reference and control purposes.

Twenty-eight normal albino rabbits weighing approximately 1.5 kg. were used as experimental animals. The animals were maintained on Wayne rabbit diet in quiet, thermostatically controlled quarters. Techniques for eliciting the delayed skin reaction, local and generalized Shwartzman, and the febrile responses followed those of Stetson (*loc. cit.*). The method of Thomas (*J. Exptl. Med.*, **104**:865, 1956) was employed for the demonstration of endotoxin potentiation of epinephrine. Each of the several local phenomena was typically observable as a 1–3-cm. skin lesion.

Delayed local inflammatory reaction.—Intradermal injection of 0.4 ml. streptococcal lysate into each of five rabbits elicited characteristic erythema, induration, and edema, which was readily demonstrable at 24 hours and thus consistent with the observations of Stetson (*loc. cit.*). Saline-injected sites were non-reactive.

Local Shwartzman.—Intravenous injection of 200 μg. *E. coli* endotoxin at the height of the skin reactions in these same rabbits caused the development of severe hemorrhagic necrosis at the initially reactive sites.

Generalized Shwartzman.—One of five rabbits prepared with intramuscular injections of cortisone acetate (35 mg/day) for 3 days developed cortical necrosis following provocation with 2.0 ml. streptococcal lysate intravenously.

Febrile response.—Four animals injected intravenously with either 2.0 ml. lysate or a washed cell suspension and followed for 4 hours showed a significant elevation of body temperature when compared with four animals injected with pyrogen-free, physiologic saline.

Epinephrine potentiation.—Five rabbits injected intravenously with 2.0 ml. lysate and then intradermally with 0.2 ml. physiologic saline at one site and 0.2 ml. epinephrine (1:1000) in physiologic saline at a second site uniformly developed severe hemorrhagic necrosis at the epinephrine site within 24 hours. Reactions were identical with those observed in an equal group of rabbits similarly tested with 200 μg. *E. coli* lipopolysaccharide.

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The Endotoxic Activity of Human Saliva

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The lipopolysaccharide somatic antigens of Gram-negative bacteria are commonly known as "endotoxins" and produce characteristic physiologic disturbances in animals. Similar biologic properties are found in certain streptococci and in various plant and animal tissues (C. A. Stetson, *Cellular and Humoral Aspects of the Hypersensitive States*, p. 442 [New York: Hoeber-Harper, 1959]). Since it has been repeatedly suggested that endotoxins may play a role in the pathogenesis of gingival and periodontal disease, it seemed desirable to confirm their presence in the mouth and oral fluids by a uniquely identifiable and sensitive means.

Advantage was therefore taken of the fact that endotoxin specifically and dramatically potentiates the necrotizing action of epinephrine as much as one thousand fold (L. Thomas, *J. Exptl. Med.*, **104**:865, 1956). Intradermal epinephrine produces extraordinary hemorrhagic necrosis in the skin of rabbits receiving an intravenous injection of endotoxin at any time during the preceding 4 hours.

Previous intravenous injections of non-toxic materials do not prepare animals for this response. Substances with extraneous irritative properties, e.g., acid or alkaline solutions, likewise do not induce this response. Intradermal injection of epinephrine alone into normal rabbits in repeated amounts as large as 200 μg. is accompanied only by a transient local erythema.

Preparative Controls		Saliva of Healthy Subjects					Saliva of Periodontal Patients	
Non-injected	Endotoxin*	1	2	3	4	5	6	7
0/5.....	5/5	1/10	2/5	4/5	2/5	3/5	2/8	5/5

* Difco Laboratories, Detroit 1, Michigan.

Fifty-three albino rabbits, weighing between 1.5 and 2 kg. and maintained on Wayne rabbit diet, were employed in testing the individual samples of saliva secured from five subjects with good oral health and from two patients with moderate to advanced periodontal disease. Each specimen of whole, unfiltered saliva containing its natural flora was secured by unstimulated expectoration into cellulose acetate tubes. All specimens were stored at 1° C. for approximately 1 week. The resistance of endotoxins to enzymatic digestion and their indifference to pH changes made further manipulation of the saliva unnecessary.

All animals were injected intradermally in the shaved abdomen with 0.2 ml. physiologic saline (0.15 M NaCl) at one site and 0.2 ml. epinephrine (1:1000) in physiologic saline at a second site. The animals in each experimental group were prepared immediately beforehand by intravenous injection of 1 ml. of an individual saliva specimen. Five control animals were prepared by the injection of 200 μg. *E. coli* (026:B6) lipopolysaccharide* in physiologic saline. Five additional control animals were not given any preparative intravenous injection. All animals were examined for the presence or absence of local lesions 24 hours after intradermal injection in accordance with the method of Thomas (*loc. cit.*). Variability in size of lesions (1–3 cm.) among the individual animals was disregarded. In the tabulation the number of animals employed appears in the denominator, and the number of animals developing hemorrhagic necrosis in the numerator.

It is clear that all specimens of saliva exhibited some endotoxic activity. Observed variation in response may be attributed to variability in animals and heterogeneity of saliva samples, as well as to real differences in concentration of the active agent. It is of interest that the patient with advanced periodontal disease supplied saliva which induced hemorrhagic necrosis in all five of the tested animals. The pathogenetic significance of salivary endotoxic activity is currently under study.

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Method of Pure Parotid Saliva Collection without Cannulization

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Samples of parotid saliva can be collected by a variety of techniques. W. A. Curby (*J. Lab. Clin. Med.*, **41**:493, 1953) and J. E. Finesinger and G. L. Finesinger (*J. Lab. Clin. Med.*, **23**:267, 1937) have constructed universal adapters which are both difficult and expensive to fabricate, thus limiting their use. A modification of such adapters has many advantages over those previously reported. It is (1) simple in construction, (2) inexpensive to fabricate, (3) easy to adapt on patients of all ages, and (4) can be conveniently sterilized by both boiling and cold disinfectants. In all cases no trauma resulted, despite the fact that the appliance covered the parotid duct for periods up to 90 minutes. The parotid saliva collector was constructed in the following manner.

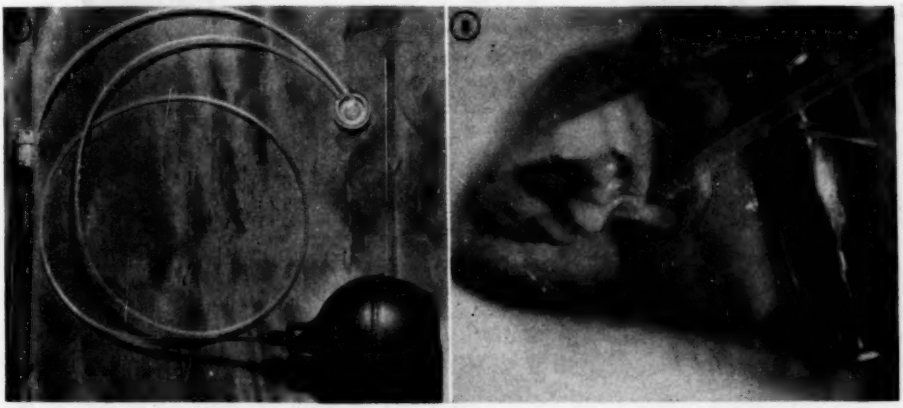

FIG. 1.—*A*, the completely assembled and ready-to-use parotid saliva collector. *B*, the saliva-collecting appliance in place covering the opening of the parotid duct.

A Lucite cap was turned down and reamed on a lathe. Polyethylene suction tubing* with an inside diameter of 0.066 inch and an outside diameter of 0.095 inch was used. The collection tubing consisted of P-E No. 260 tubing with an I.D. of 0.070 inch and an O.D. of 0.110 inch. A suction system was made by attaching a small rubber pipette bulb to a 15-gauge hypodermic needle. This was then connected to the suction chamber with polyethylene tubing. The assembled parotid collector is shown in Figure 1, *A*.

Saliva was collected in the following way: The mucous membrane of the patient's cheek was dried with a sterile gauze. The orifice of Stenson's duct opposite the maxillary second molar tooth was located. The pipette bulb was squeezed; the adapter was centered over the orifice and the bulb released, creating the suction needed to hold the appliance in place (Fig. 1, *B*). After the patient stimulated the flow of saliva by chewing paraffin on the same side as the duct being sampled, the tube coming from the collection chamber was placed in a test tube so that a specimen could accumulate.

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* Clay-Adams tubing No. 260.

Modified Method of In Vitro Production of Salivary Deposits

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The apparatus and system reported for studying artificial calculus deposition in vitro (S. W. Leung, *J. Periodont.*, **28**:217, 1957) were modified to permit simpler examination and evaluation of initial stages of deposits per single saliva specimen.

The incubator (Fig. 1) is a wooden frame, 21 × 21 × 17 inches, covered by a plastic sheet, except for the hinged glass front, and lined inside with aluminum foil. Each plastic container holds 5–10 cc. of one saliva specimen, covered with yellow base-plate wax perforated for a wire holding a glass slide, ¼ × 1 inch, suspended in the saliva.

Unstimulated saliva was collected by subjects' expectoration augmented by aspiration. Fresh specimens were placed daily in the rocking box at one time. The glass slides were examined briefly while wet under a stereomicroscope after 24 and 48 hours. After 72 hours, slides were air-dried for

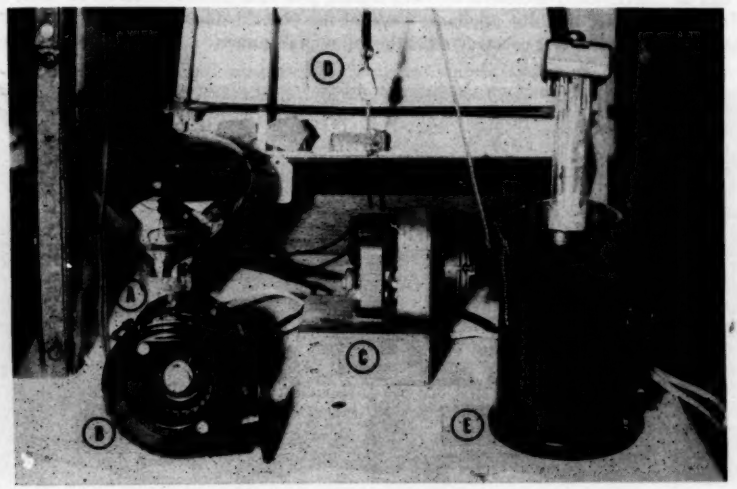

FIG. 1.—Items of equipment contained in incubator: *A*, 250-watt infrared bulb (heat source); *B*, electric circulating blower; *C*, 1-rpm fractional horsepower motor with eccentric pulley; *D*, rocking box holding covered saliva samples (capacity = 40 containers); *E*, water container with immersion heater. Not shown is Thyratron relay which, with a bimetallic regulator, is attached to the heat source, constituting the temperature-regulating system.

final examination, without staining. Classifications were as follows: "film," translucent, thin, relatively homogeneous; "plaque," opaque, heterogeneous, containing particles; "calculus," perceptibly crystalline. Deposits were compared with specimens of actual plaque and calculus scraped from the subjects' teeth and kept separately in the incubator. Spectrophotometric graphs of plaque and calculus specimens deposited in vitro were closely similar to graphs of analogous specimens collected intraorally.

After 72 hours of processing, most saliva specimens ceased to deposit. Ninety per cent of 290 specimens of 11 subjects yielded some deposits within 72 hours. The quality and quantity of deposits varied per specimen. One hundred and forty-eight specimens (51 per cent) deposited some calculus crystals. However, none of the 10 specimens of the 2 subjects who were of prepuberty age deposited crystals of calculus.

This work was supported in part by a grant from Warner-Lambert Pharmaceutical Company, Morris Plains, New Jersey.

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Silicone Rubber as a Resilient Denture-Base Material

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This report deals with the technical considerations regarding the use of certain medical-grade silicone rubber as a resilient denture-base material alone or in conjunction with polymethyl methacrylate.

Paffenbarger and Bowen (*J.A.D.A.*, **56**:788, 1958) reported that "polymethyl methacrylate has not had a serious challenger for first place as a denture-base resin since it was introduced in the late 1930's, and that no development to date, with the possible exception of epoxy resins, would indicate its displacement." They further stated that "soft relining resins will become more extensively used as they improve in quality." The search for better-quality soft materials has prompted this work.

Silicone rubber was chosen because it will remain resilient for an indefinite perod of time and is particularly inert to body fluids and tissues. It is odorless, tasteless, and can be easily molded. When vulcanized it possesses good dimensional stability.

Silicone rubber forms can be made following the same general technique used in the processing of methyl methacrylate, with minor modifications. On a dental stone cast,* the pattern is formed in base-plate wax, which is invested in the same type stone in a denture flask which can be bolted together when closed. Upon flushing the wax from the mold, a liquid foil separating agent is applied to the mold surfaces, and the silicone rubber is introduced into the mold. This translucent material requires no mixing, as it is in a semisoft state when received from the manufacturer. The flask is closed in the bench press to a metal-to-metal contact, opened, flash-trimmed, and closed. When the mold cavity is completely filled, the flask is then bolted together and placed in an air-circulating dry-heat oven for 3 hours at 300° F. Upon completion of this molding time, the flask is bench-cooled to handling temperature and the rubber form removed. This is then returned to the oven free of the flask and stone, where it remains for an additional time of 16 hours at 480° F.

Two types of medical-grade silicone rubber have been used in five situations which are presently being clinically evaluated: (A) Silastic S-2055† and (B) Silastic X-3-0146,‡ S-2055 being the firmer of the two. The five experimental conditions were as follows: (1) Full palatal coverage of S-2055, 3 mm. thick, reinforced across intertuberosity area with a narrow piece of wire mesh,§ imbedded in the rubber during packing. (2) Full palatal coverage of methyl methacrylate denture base material with hollow obturator of X-30146 extending into the antrum through a surgically induced opening in the hard palate. These two materials were joined together with a silicone medical-grade adhesive Q-3-0149.|| (3) Same as above except that obturator was of S-2055. (4) Same as 2, except that base was of S-2055, hollow obturator was of X-3-0146; again these two materials were joined together with Q-3-0149. (5) Silicone rubber palate of S-2055 reinforced with wire mesh. The solid obturator portion was made of X-3-0146. The S-2055 and X-3-0146 were fused together during the molding stage.

Without doubt time will indicate improvements which will advance this early work. However, it is conceivable that this material could be incorporated into prosthetic appliances fabricated for the patient with either a congenital or surgically induced cleft palate. Beyond this phase, work is being carried out attempting to incorporate denture teeth into base material of silicone rubber.

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* Vel-Mix Stone, Kerr Dental Mfg. Co., Detroit, Michigan.

† Silastic S-2055, medical-grade silicone rubber, Dow Corning Corp., Midland, Michigan.

‡ Silastic X-3-0146, medical-grade silicone rubber, Dow Corning Corp.

§ Vitallium wire mesh, Austenal Dental Division, Chicago, Ill.

|| Medical-grade silicone adhesive Q-3-0149, Dow Corning Corp.

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